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Antimicrobials and Agriculture

The Proceedings of the 4th International Symposium on Antibiotics in Agriculture: Benefits and Malefits

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PREFACE

The Easter Schools in Agricultural Science began in 1953 with the one on Micropedology, organized by Professor Gordon Hallsworth. This success encouraged the following Easter Schools and led to two on Antibiotics in Agriculture. The first of these—originally envisaged for 1956 or 1957—was anticipated by the First International Conference on Antibiotics in Agriculture, which was held in Washington in 1955 and published in 1956 (as publication 297 of the National Academy of Science-National Research Council). The Americans decided not to sponsor another such conference and so the Second International Conference on Antibiotics in Agriculture was held at Sutton Bonington in 1962 (as the 9th Easter School) and published by Butterworths in 1962. The success attached to this effort and the continuing one of Easter Schools led to the prospect of a Third International Conference and 25th Easter School in Agricultural Science on Antibiotics in Agriculture. This was based on the view that the updating of earlier topics could be regarded as either a good thing *per se* or as losing some appeal by virtue of repetition.

The 1976 programme was designed to meet these views by updating the 1956 and 1962 volumes and to introduce a new aspect. Accordingly, contributions (and topics) were invited to give 'special reference to synergism'. This volume was published in 1977 by Butterworths. Those attending the 25th Easter School considered that another should be held under the title of 'Antibiotics in Agriculture: Benefits and Malefits', a suggestion put forward by Sir James Howie.

In the event, the 1983 meeting was held as the Fourth International Symposium on Antibiotics in Agriculture—with the considerable support listed in the acknowledgements—and is published, by Butterworths, under the agreed title 'Antimicrobials and Agriculture'.

Sir James Howie chaired a short final session of the symposium, on the topic Where to Now? It was then agreed (1) that the 'spine' of Soil-Plant-Animal-Feed-Food-Public Health had provided a sound framework to discuss Antibiotics and Antibiosis in Agriculture, (2) that, as far as the future could be seen, it would be an excellent idea to have a 5th Symposium, possibly about 1988, (3) that the present editor, his possible successor, or Professor Alan Linton, be responsible, (4) that of the various suggestions on a theme,

the one suggested by Professor Alasdair Steele-Bodger received general approval: 'Antibiotics and Agriculture: Remits and Responsibilities'.

Malcolm Woodbine

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To my colleagues Dr Alan Seaman, Mr David Fowler, Mr John Rosillo, Mrs Caroline Walker, and to my students: post-graduates Hisham Bashir, Munim Amin; undergraduates Joanne Morley, Georgina Simpson, Sarah Tullett, Bill Wadsworth; to our domestic, library and administrative colleagues, especially to Ms Jeanette O'Neill for her admirable secretarial support; to Ms Doreen Borrows who acted as Symposium Secretary; and to my wife Hilda both for her general support and help with the references and indexing with which my daughter Barbara was also associated. Many thanks also to the staff at Butterworths for their help in the preparation of the volume.

PROLOGUE

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In many ways, this symposium on the benefits and disadvantages of antibiotics in agriculture is well timed. The antibiotic era—if one discounts the sulphonamides as antibiotics—has now lasted for about 40 years and the benefits and disadvantages of these agents are fairly clear for all to see. On the one hand there are undoubted advantages: agents now exist which do indeed have enormously valuable properties and many infectious conditions are largely controlled—both in man and domestic animals—by their use. Moreover, treatment is much more effective and rapid, with clear advantages in morbidity and the incidence of unwanted side-effects.

Against this background there are some fairly notable disadvantages. Resistant populations of bacteria have emerged in many cases and these have sometimes caused severe clinical problems. Perhaps few of these are actually life-threatening, but often they delay recovery and lead to unwanted complications.

Although it is clear that antibiotics have their advantages and disadvantages, it is often rather difficult to decide where the balance lies for any one agent. If one goes to meetings on the newer β -lactam antibiotics, for example, there is much talk of resistant organisms and β -lactamase production. Despite this, however, very large amounts of benzyl penicillin and of phenoxymethyl penicillin are still sold and are effective 40 years or so after their first use. Contrariwise, most of the new β -lactams are primarily for hospital prescription and used to treat a minority of infections.

In fact, when one considers the occasions when the well-established 'old' penicillins do not work, one commonly finds the infections in the very young, in the very old, or in the compromised. Thus, there is a hint here that the problems lie not so much with the organisms and their possible resistance, but rather with the patient and his or her relative inability to marshal the necessary defences to clear the infection.

In fact, when one reviews the position—and this is particularly clear for the β -lactams—one finds a situation where one has a range of generally rather effective 'old' compounds each with its typical therapeutic role and then a large number of newer introductions, all very similar in properties, and all seeking a niche in the market. The fact that the advantages of a particular compound are only marginal makes it ever more difficult for the physician to

4 *Prologue*

decide which agent to use; in the face of a battery of advice, he tends to throw in his hand and remain wedded to a well-tried, but often marginally less good, alternative.

The fact that it is an impairment of the defences of the host which seems to be of the greatest importance, now that we have a large range of effective antibiotics, points the way to the future as far as research and development of new anti-infectives is concerned. What seems to be needed is the agent to augment the action of the 'old' antibiotics by potentiating the defence mechanisms compromised in the host. Perhaps this end can be achieved by an enhancement of the immune response, or by the potentiation of phagocytosis or by some other alteration of the normal protective measures of the host.

If the future of antibiotic therapy is indeed destined to be part of a combined therapy—part antibiotic and part modulator of the defence mechanisms—it is clear that much work needs to be done; and it is in this connexion that one has reached a critical point. The sort of research to generate the new compounds will be of a type very different from that which evolved the antibiotics we now know. In general, synthetic chemistry will be less involved, and modern biology—immunology, cell biology, development—will become paramount.

In fact, this need to alter the point of research attack so radically will make for major difficulties in the pharmaceutical companies. One can readily see at least three. First, the type of research worker needed will change and the skills which seemed of paramount importance in the past will be needed less. Secondly, the direction of the research effort will give rise to difficulties. In many companies the senior research directors have reached their positions from a background of organic chemistry—a testimonial to the routes to successful products in the past. Such people—often excellent and gifted researchers—find the imperatives of biological research hard to grasp; and it is often difficult for a biologist to urge a successful case for a new research project in such an environment. Perhaps it is not accidental that several recently appointed research directors of major companies are biologists introduced from academia rather than promoted from within the company.

Almost certainly the greatest difficulty at this present critical juncture, however, is to know precisely where to concentrate one's research effort. If one has the objective of supporting conventional antibiotics with agents which enhance the normal defences of the host, where does one start? Too little is known to be able to focus one's research; yet the resources available—even to industry—are simply incapable of covering all the necessary areas.

It is against this background that one feels that university research may soon become, once again, of paramount importance to the pharmaceutical companies. In the recent past, when the semisynthetic modification of existing nuclei was of supreme importance, most of the necessary effort and expertise were available within the research departments of the companies. Now there is a real need for a much greater insight into the fundamental biology of the infectious process if the development of new agents is going to be achieved, and this is likely to arise from work in academic departments. Indeed the transition, which is the central point for discussion in this brief prologue, gives major chances for the universities of the world. Their efforts will be of crucial importance if we are to move successfully into the next phase of the antibiotic era.

BETA-LACTAMASE INHIBITORS: BIOCHEMICAL PROPERTIES AND BACTERIOLOGICAL APPLICATION

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The interest in inhibitors of the β -lactamase enzymes (penicillinases and cephalosporinases, etc.) arises from their importance as resistance determinants for β -lactam antibiotics. There are several mechanisms by which bacteria can be resistant to the lethal effects of penicillins and cephalosporins, but probably the most important involves destruction of the essential β -lactam ring of these molecules by β -lactamases in the bacterial cell wall. These enzymes are surprisingly widely occurring and many different types exist, several of the most important being plasmid mediated.

Much research effort has been and is still directed to finding members of the β -lactam class of antibacterials which have stability to β -lactamases. However, it has not proved easy to achieve stability to a wide range of these enzymes while retaining previously existing desirable features. This situation has encouraged the search for β -lactamase inhibitors which might enhance the activity of labile compounds against resistant strains. For such inhibitors to be of therapeutic use they must, of course, be able to protect the labile compound from those β -lactamases which most readily attack it.

The β -lactamase inhibitors which were first found, for example methicillin and cloxacillin, did not meet all of the above criteria. Much more promising results have been obtained with recent compounds such as salts or derivatives of clavulanic acid and penicillanic acid sulphone.

This chapter reviews the various β -lactamase inhibitors and discusses our observations on some of the β -lactamases of veterinary isolates of bacteria, their inhibition by potassium clavulanate and its ability to potentiate the activity of amoxycillin against these bacteria.

Mechanism of penicillin resistance

The three main mechanisms by which bacteria may be resistant to the action of penicillins are illustrated in *Figure 2.1*. First, the target site enzyme (transpeptidase) may have structural features which make it less readily inhibited by penicillin than is the case in sensitive cells. This may be the situation in methicillin-resistant staphylococci (Hayes *et al.*, 1981). Secondly, the outer layers of the cell wall may be of such a composition as to prevent or seriously

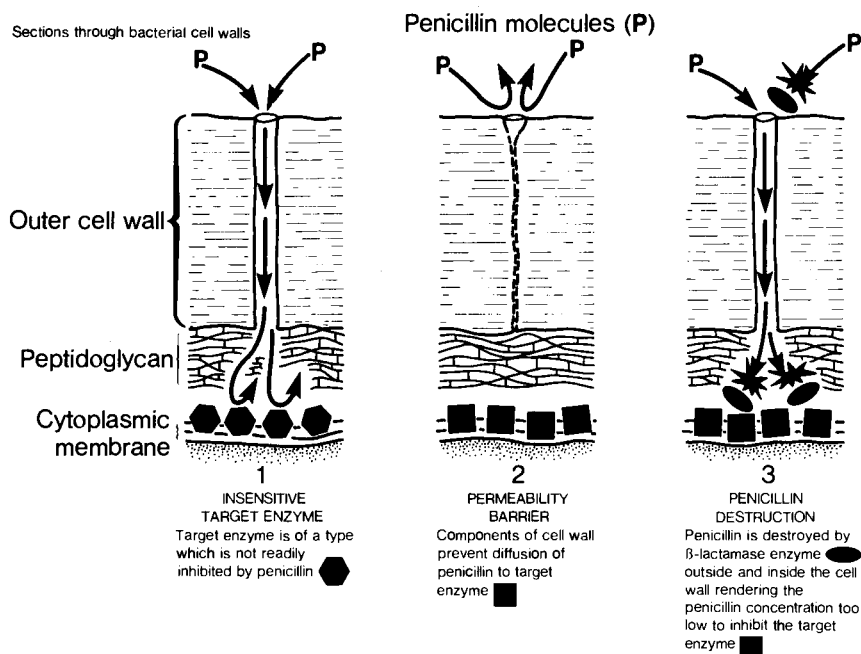


Figure 2.1 Mechanisms of resistance to penicillins

retard the penetration of penicillin molecules to the target site enzyme. This mechanism probably explains why benzylpenicillin has poor activity against Gram-negative bacteria in spite of its good affinity for the penicillin-binding proteins (Curtis *et al.*, 1979).

The third mechanism involves destruction of penicillin molecules by bacterial enzymes called β -lactamases. These enzymes are usually located in a region of the cell wall just outside the cytoplasmic membrane. They are thus in an ideal position to prevent the build-up of an inhibitory concentration of penicillin at the target site enzyme. In some bacteria, for example certain strains of *Staphylococcus aureus*, the β -lactamase is also liberated from the cell (exocellular enzyme).

Enzymes which destroy penicillins and cephalosporins—the β -lactamases

The β -lactamases catalyse the hydrolysis of the β -lactam ring of penicillins and cephalosporins to give products devoid of antibacterial activity. Sometimes the names 'penicillinase' and 'cephalosporinase' are used for these enzymes, depending on the substrate, but this can be confusing as many of the enzymes having penicillinase activity can also attack cephalosporins and vice versa. Amoxycillin is unstable to several different types of β -lactamases, the product of the reaction being an inactive penicilloic acid (see Figure 2.2).

The presence of a β -lactamase in a bacterial culture can be demonstrated by disrupting the cells with ultrasonics, mixing the resulting crude enzyme

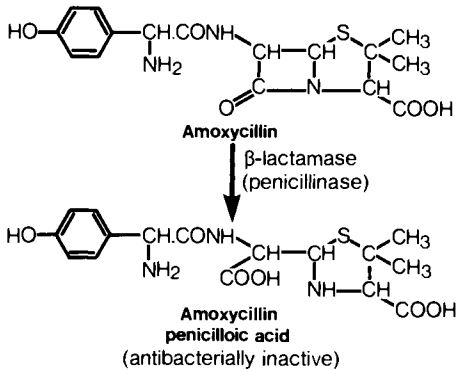


Figure 2.2 Action of bacterial β -lactamase on amoxycillin

preparation with the penicillin or cephalosporin under investigation and following the reaction spectrophotometrically or by bioassay (Sykes and Matthew, 1979). The enzymes are produced by many pathogenic bacteria, as indicated in *Figure 2.3*. The various types can only be differentiated by a range of tests including substrate profiles, the effect of inhibitors and isoelectric focusing in polyacrylamide gels. A guide to the types of enzyme in Gram-negative bacteria has been prepared by Sykes and Matthew (1976). Matthew (1979) has described the properties and distribution of the various plasmid-mediated β -lactamases. Further information about these enzymes can be found in Hamilton-Miller and Smith (1979).

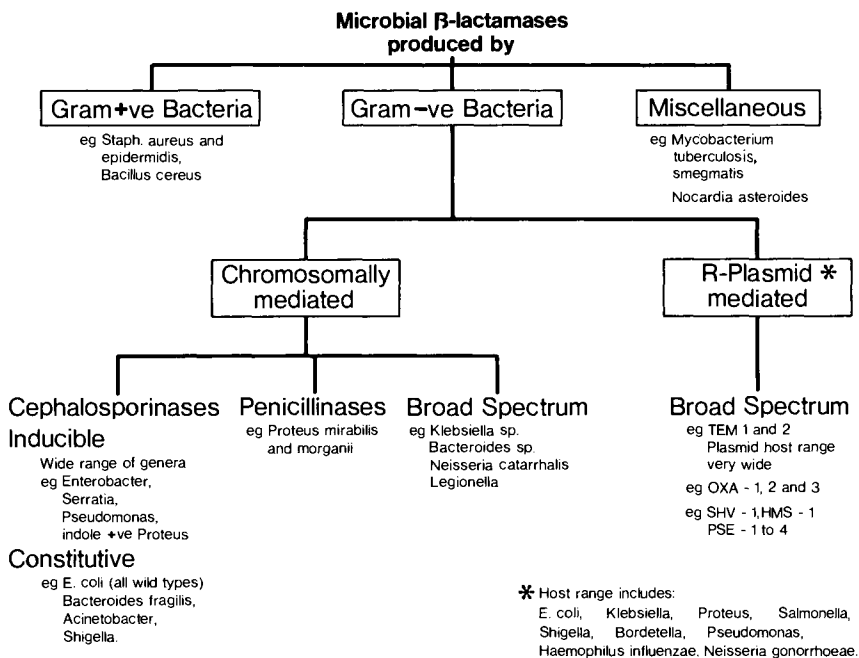
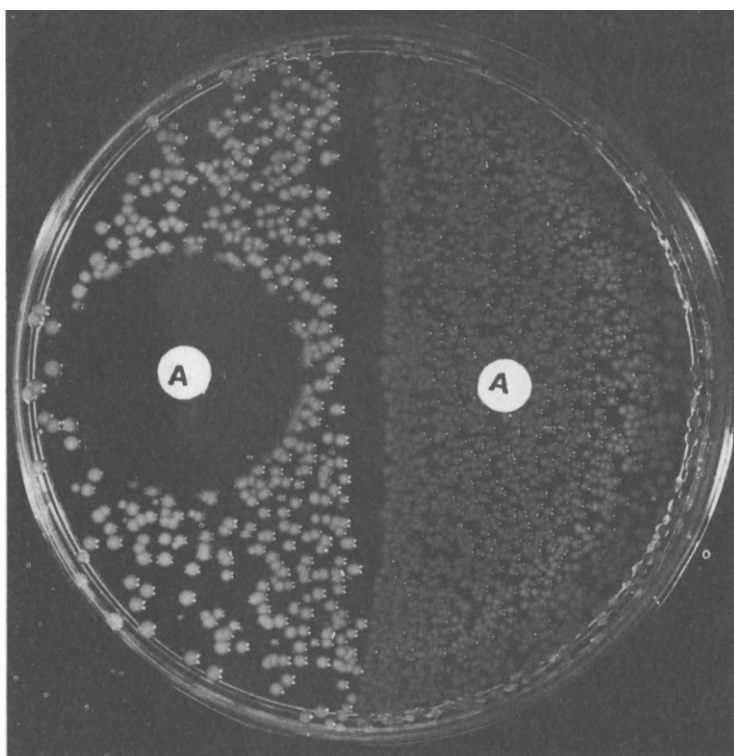


Figure 2.3 Beta-lactamase-producing bacteria

Beta-lactamase-mediated penicillin resistance and ways of overcoming it

Production of β -lactamase by a bacterium usually confers very considerable resistance to a penicillin (or cephalosporin) when it is a substrate for the enzyme. The effect can be demonstrated by placing paper discs impregnated with antibiotic on agar plates seeded with the test bacterium. In *Figure 2.4*, a 20 μ g amoxycillin disc gave a large zone of growth inhibition when contacted with *Escherichia coli* (Abbotstown), but no zone was seen when a similar disc was placed on the culture after transferring to it a resistance plasmid mediating the TEM-1 β -lactamase. The density of the inoculum was slightly different for the two cultures, but this was not responsible for the dramatic difference in zone diameters. The effect of production of β -lactamase is also seen when the minimum inhibitory concentration (MIC) is determined in agar or in broth. The MIC value for amoxycillin may increase to over 1000 μ g/ml against a strain of *E. coli* which produces the TEM β -lactamase. However, intermediate and variable results may be obtained, depending on the amount of β -lactamase produced and the rate at which it hydrolyses the antibiotic being tested.



E. coli *E. coli*
(Abbotstown) (Abbotstown) R⁺ TEM-1

Figure 2.4 Effect of presence of the plasmid-mediated TEM-1 β -lactamase on sensitivity of *Escherichia coli* to an amoxycillin disc (20 μ g) (by courtesy of B. West, Beecham Pharmaceuticals)

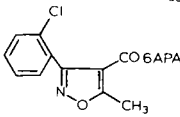
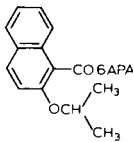
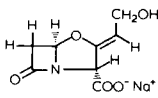
Penicillins and cephalosporins have been selected which have structural features conferring stability to certain β -lactamases. Unfortunately it has not been possible so far to find a broad spectrum, orally absorbed penicillin which also has stability to a wide range of β -lactamases. For example, the penicillin cloxacillin has stability to a wide range of β -lactamases including staphylococcal, but it does not have useful activity against Gram-negative bacteria. Another approach to the problem involves protecting the β -lactamase labile antibiotic by addition of a β -lactamase inhibitor. In this way the useful properties of established β -lactam antibiotics are not lost, but can be extended to include activity against many otherwise resistant strains of bacteria.

Penicillins, penicillanic acid derivatives and cephalosporins as β -lactamase inhibitors

The first compounds to be found with specific β -lactamase inhibitory activity were the β -lactamase-stable penicillins such as methicillin, the isoxazolympenicillins (cloxacillin, flucloxacillin, dicloxacillin) and cephalosporins, for example cephalosporin C and cephoxazole (for review, see Cole, 1979). Compounds such as cloxacillin (Table 2.1) and cephoxazole show inhibitory activity against the β -lactamases of *Enterobacter cloacae* and *Pseudomonas aeruginosa* and the chromosomally controlled enzyme of *E. coli*, but are not very effective against the important plasmid-mediated enzymes or staphylo-

Table 2.1 COMPARISON OF THE β -LACTAMASE INHIBITORY ACTIVITIES OF CLOXACILLIN, BRL 1437 AND CLAVULANIC ACID

Source of
 β -lactamase

	<i>I</i> ₅₀ value ^a (μ g/ml) for:		
			
	Cloxacillin	BRL 1437 ^b	Sodium clavulanate
<i>Staphylococcus aureus</i> MB9	> 40	> 40	0.02
<i>Escherichia coli</i> R ⁺ TEM	> 40	0.21	0.08
<i>E. coli</i> JT414 (chromosomal)	0.14	16	50
<i>Proteus mirabilis</i> C889	> 40	13	0.01
<i>Klebsiella aerogenes</i> A (<i>pneumoniae</i>) ATCC 15380	> 40	0.88	0.01
<i>Enterobacter cloacae</i> NCTC 10005	2.5	4	50
<i>Pseudomonas aeruginosa</i> A (Sabath)	2.2	14	> 50

^a Concentration of inhibitor required to give 50% inhibition of the rate of hydrolysis of benzylpenicillin (1 mg/ml) after incubating the inhibitor with the β -lactamase for 15 min at 37°C, pH 7. The concentration of each β -lactamase preparation was such that, in the absence of inhibitor, about 50% hydrolysis of benzylpenicillin occurred in 30 min at pH 7 and 37°C. All inhibitors were used as their sodium salts.

^b BRL 1437 is 2-isopropoxy-1-naphthylpenicillin.

6APA = 6-aminopenicillanic acid.

coccal β -lactamase. These inhibitors act mainly by the competitive mechanism. Antibacterial synergy between amoxycillin and flucloxacillin was shown by Comber, Merrikin and Sutherland (1979) for strains of Gram-negative bacteria which produced chromosomally mediated β -lactamases. Kritzing (1979) has reported clinical trial data for such a combination (Suprapen, Beecham Pharmaceuticals) and more recently the combination of cephalexin with flucloxacillin (Flucexin, Shering) has been subjected to clinical trial (Privitera, Bonino and Del Mastro, 1981).

Unlike the isoxazolylpenicillins, alkoxy-substituted penicillins can have good inhibitory activity against the plasmid-mediated TEM type of β -lactamase and also the enzyme produced by *Klebsiella aerogenes* (*pneumoniae*). 1-Naphthyl penicillin is a substrate for the TEM enzyme, but introduction of a methoxy at position 2 makes it an inhibitor. Extending the length of the alkoxy side-chain improves the inhibitory action, the best compound in the series being BRL 1437 which has a 2-isopropoxy-1-naphthyl side-chain (Cole, Elson and Fullbrook, 1972). BRL 1437 has activity against several β -lactamases, but it does not inhibit the staphylococcal enzyme to which it is stable (Table 2.1). In this respect it resembles the isoxazolylpenicillins. Enhancement of the antibacterial activity of ampicillin and cephalothin by BRL 1437 has been demonstrated, but high concentrations were required (Greenwood and O'Grady, 1975).

In recent years most of the new cephalosporins which have improved stability to β -lactamase have been investigated for their β -lactamase inhibitory action. Compounds such as cefuroxime, ceftizoxime, cefonicid, cefotriaxon, cefotetan and 7 α -methoxy-substituted cephalosporins such as cefoxitin and moxalactam (oxacephem) have been shown to have good competitive or non-competitive activity against cephalosporin-hydrolysing β -lactamases such as that produced by *Ent. cloacae* (for review, see Cole, 1981). Cephalosporins containing a dithiolane side-chain in the 7 β position have recently been described as having very high inhibitory activity against the cephalosporinases of *Proteus* species and to potentiate the activity of cephaloridine against cephaloridine-resistant strains of *Proteus*, *Enterobacteria* and *Serratia* (Ohya, Miyadera and Yamazaki, 1982).

English *et al.* (1978) reported that penicillanic acid sulphone (Pfizer No. CP45899, sulbactam) inhibited the β -lactamases of both Gram-positive and Gram-negative bacteria and was able to extend the antibacterial spectrum of β -lactam antibiotics. The ability of the compound to potentiate the activity of ampicillin-resistant strains of *Staphylococcus*, *Haemophilus influenzae* and *Bacteroides* was described by Retsema, English and Girard (1980). Other studies on this compound have been reviewed, including comparisons with clavulanic acid (Cole, 1981). The pivaloyloxymethyl prodrug derivative (CP47904) has improved oral absorption (Foulds *et al.*, 1980) and mutual prodrugs have been made by chemically linking sulbactam via a hydrolysable ester bridge to ampicillin, to give VD-1827 (Figure 2.5) and to mecillinam to give VD-1825 (Baltzer *et al.*, 1980). These compounds provide improved oral absorption of both components. The compound resulting from the linking of ampicillin with sulbactam has been called sultamicillin and its properties described in a series of papers given at the American Society for Microbiology ICAAC Conference, in 1982 (see Stam *et al.*, 1982, and subsequent abstracts).

The 2 β -chloromethyl analogue of penicillanic acid sulphone (BL-P2013)

has been reported by workers at Bristol Laboratories to have β -lactamase inhibitory properties similar to that of sulbactam (Gottstein *et al.*, 1981). The data presented by these authors indicated that both compounds were poorer than clavulanic acid at protecting a cephalosporin from the destructive action of several β -lactamases. *In vitro* and *in vivo* antibacterial synergy data were also given.

Penicillanic acids with a halogen substituent in the 6- β position, that is projecting above the plane of the β -lactam ring, have β -lactamase inhibitory activity. The first reports on this subject referred to the formation of β -lactamase inhibitory activity in a solution of 6 α -bromopenicillanic acid as it

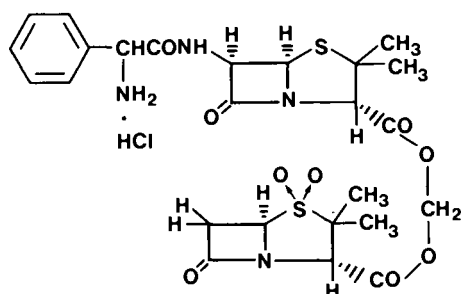


Figure 2.5 Structure of VD-1827, sultamicillin, a mutual prodrug of ampicillin and penicillanic acid sulphone (after Baltzer *et al.*, 1980)

epimerized to the 6 β -bromo form (Pratt and Loosemore, 1978; Knott-Hunziker *et al.*, 1979). The 6 β -iodo (Pfizer, UK 38006) and 6 β -chloropenicillanic acids (Kemp *et al.*, 1980; Daehne, 1980) have also been reported to have β -lactamase inhibitory activity. Moore and Brammer (1981) demonstrated the progressive inhibitory properties of the β -iodo compound and determined its ability to potentiate the *in vitro* activity of various penicillins and cephalosporins and the *in vivo* activity of bacampicillin. Wise, Andrews and Patel (1981) have compared the 6 β -bromo and 6 β -iodo compounds with clavulanic acid and sulbactam for their ability to potentiate the activity of ampicillin against a range of ampicillin-resistant bacteria *in vitro*. Clavulanic acid was generally the most active compound, followed closely by β -bromopenicillanic acid which was slightly more active than β -iodopenicillanic acid and more active than sulbactam. The β -bromo and β -iodo compounds were a little better than clavulanic acid for *Haemophilus influenzae* carrying the TEM β -lactamase.

Recently, 6-acetylmethylene penicillanic acid (Ro 15-1903) has been described as a potent progressive inhibitor of chromosomally and R-factor-mediated β -lactamases (Arisawa and Then, 1982). Its ability to potentiate the activity of ampicillin against resistant bacteria is similar to or slightly less than that of clavulanic acid (Angehrn and Arisawa, 1982). Ro 15-1903 appears not to be active in mice by the oral route, but the pivaloyloxymethyl ester (Ro 15-1315) is said to be more stable and of potential interest for oral administration (Arisawa and Then, 1982; Angehrn and Arisawa, 1982).

A comparison of the activities of some substituted monocyclic β -lactams

against staphylococcal β -lactamase has been made by Isaacs, Sunman and Reading (1982). The antibiotic azthreonam belongs to the same class of compounds and is related to the microbial metabolites called monobactams. Azthreonam has been reported by Bush, Freudemberger and Sykes (1982) to have good inhibitory activity against *Ent. cloacae* P99 β -lactamase.

Microbial metabolites as β -lactamase inhibitors

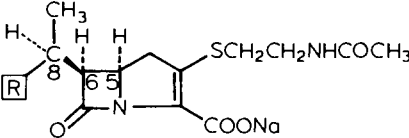
Among the most active β -lactamase inhibitors found in microorganisms are clavulanic acid and members of the olivanic acid family of antibiotics (Brown *et al.*, 1976). Clavulanic acid is discussed in detail in the next section.

The olivanic acids are made in low yields by *Streptomyces olivaceus*. Like the penicillins and cephalosporins these compounds contain a β -lactam ring system, but it is fused to an unsaturated 5-membered ring containing carbon in the place of the sulphur atom of the penicillin molecule. In view of the structure of this nucleus, these compounds are sometimes called 'carbapenems'. Included in this group, in addition to the olivanic acids (Beecham) are the thienamycins (Merck), the PS5 group of compounds (Sanraku Ocean), the carpetimycins (Kowa), C19393 (Takeda), the asperenomycins (Shionogi) and SF 2103A (Meiji Sieka), all being produced by various cultures of *Streptomyces*. The substances have alkyl or substituted alkyl side-chains in position 6 and, in position 2, another side-chain containing a thioethylamine moiety in various states of oxidation and usually with an *N*-acetyl substituent, as in the olivanic acids. In SF 2103A, the 2-substituent is $-\text{SO}_3\text{H}$. Most of these substances are notable for their antibiotic activity, but they also have various degrees of β -lactamase inhibitory activity. A recent publication on carpetimycins A and B (Kobayashi *et al.*, 1982) illustrated the potent antibacterial activity of these substances and their inhibitory activity against both penicillinases and cephalosporinases.

The olivanic acids with the highest β -lactamase inhibitory activity are those with a sulphated 1-hydroxyethyl side-chain in position 6, namely BRL 17880, BRL 13902 and BRL 4550. All of these compounds are potent broad spectrum antibiotics and have high activity against a wide range of β -lactamases, the sulfoxide BRL 4550 being the most active against the TEM and *K. aerogenes* β -lactamases. When used at sub-antibacterial concentrations, the olivanic acids can potentiate the activity of penicillins such as amoxycillin against certain resistant strains of bacteria (Basker, Boon and Hunter, 1980). The marked effect of introducing the sulphate ester group can be seen by comparison of the β -lactamase inhibitory activity of BRL 22380 with BRL 17880 (Table 2.2). These compounds have the same side-chain in position 2 and the same stereochemistry, namely 5R, 6R, 8S, the 5,6 protons being in the *cis* configuration. The metabolic instability and low yields of the olivanic acids and related substances have so far precluded their therapeutic application.

Another group of microbial metabolites containing sulphate ester groups and having potent β -lactamase inhibitory activity is represented by the sulphated macrocyclic lactones izumenolide (Liu *et al.*, 1980) and dotriacolide (Ikeda *et al.*, 1981). These substances are produced by strains of *Micromonospora*. They have low I_{50} values against the β -lactamases of Gram-negative

Table 2.2 BETA-LACTAMASE INHIBITORY ACTIVITY OF THE OLIVANIC ACIDS BRL 22380 AND BRL 17880

				
R	<i>I</i> ₅₀ value ^a (μg/ml) for β-lactamases from:			
	<i>Staphylococcus aureus</i> (Russell)	<i>Escherichia coli</i> JT4 R ⁺ TEM	<i>Klebsiella aerogenes</i> E70	<i>Enterobacter cloacae</i> P99
—OH (BRL 22380)	0.1	15	10	0.03
—OSO ₃ Na (BRL 17880) (sulphate)	0.02	0.02	0.006	0.001

^a Concentration giving 50% inhibition of the rate of hydrolysis of substrate. The inhibitor was reacted with the β-lactamase for 5 min before adding nitrocefin (250 μg/ml) as substrate.

bacteria, but not that of *Staph. aureus*. They are reported to be highly toxic to mice when administered parenterally. Two β-lactamase inhibitors belonging to the alkylbenzene disulphate groups of substances, namely M-4854 I and II, were found by Yaginuma, Inoue and Mitsuhashi (1980). These substances were detected in a culture of *Chaetomella raphigera* by their inhibitory activity against the β-lactamase of *Citrobacter freundii*. All of these sulphated compounds seem to act by progressive inactivation of the β-lactamase.

Certain monocyclic β-lactam-containing antibiotics, for example the monobactams (Sykes *et al.*, 1981), have been reported as being produced by various bacteria. One of these substances, monobactam VIII, produced by *Agrobacterium radiobacter*, had pronounced inhibitory activity against the β-lactamase of *Ent. cloacae* P99, but little activity against others.

Clavulanic acid

Clavulanic acid is produced by *Strep. clavuligerus* and is the most extensively studied β-lactamase-inhibiting microbial metabolite (for review, see Cole, 1981). The bicyclic nucleus of this compound resembles that of a penicillin, but with an oxygen atom in place of the sulphur and without an acylamino side-chain in position 6 (see Table 2.1 for structure). The β-lactamase inhibitory spectrum of sodium clavulanate against a small number of β-lactamases is shown in Table 2.1 alongside corresponding data for two of the best previously described inhibitors, namely the penicillins BRL 1437 and cloxacillin (Cole, 1979). As can be seen, very low concentrations of clavulanic acid brought about 50% inhibition of the rate of hydrolysis of benzylpenicillin, by the clinically very important staphylococcal and TEM β-lactamases. Cloxacillin is not a significant inhibitor of these, whereas BRL 1437 is only active against the TEM β-lactamase. Clavulanic acid also readily inhibited the β-lactamases of *Proteus mirabilis* and *K. aerogenes* (*pneumoniae*) which were not inhibited by cloxacillin and were poorly inhibited by BRL 1437. Clavulanic acid has poor inhibitory activity against the β-lactamases

(cephalosporinases) of *E. coli* JT414, *Ent. cloacae* NCTC 10005 and *Ps. aeruginosa* A. The enzyme preparations from these organisms had low activity against ampicillin, amoxycillin, carbenicillin and ticarcillin, but very readily hydrolysed the cephalosporin, cephaloridine (Cole, 1979, and Beecham unpublished results). Clavulanic acid thus has good inhibitory activity against the β -lactamases (penicillinases) which readily hydrolyse penicillins, but poor activity against the cephalosporin-hydrolysing β -lactamase against which ampicillin, amoxycillin and particularly carbenicillin and ticarcillin already have considerable stability.

Clavulanic acid is notable for its ability to inactivate β -lactamases progressively. This is illustrated by the observation that the amount of clavulanic acid required to give 50% inhibition of enzyme activity decreases if it is allowed to react for a few minutes with the enzyme before adding substrate, as illustrated by results in *Table 2.3*. Clavulanic acid also shows competitive inhibitory activity because of its high affinity for the enzyme. The competitive phase of inhibition is characterized by K_i values in the region of 0.3–1.4 $\mu\text{mol/l}$ for a range of R-factor and chromosomally mediated β -lactamases (Labia *et al.*, 1980).

The two properties of competitive inhibition and progressive inactivation of the β -lactamase can be seen when the rate of hydrolysis of a labile penicillin is followed in the presence of clavulanic acid. There is an immediate slowing down of the rate of hydrolysis (competitive inhibition), but this rate continues to slow down until the reaction stops (enzyme inactivation). How long it takes for the reaction to stop depends on the amounts of β -lactamase and clavulanic acid present. These results are in marked contrast to those obtained with 2-isopropoxy-1-naphthyl penicillin (BRL 1437). Against the β -lactamases of *Klebsiella* and *E. coli* R⁺TEM, this compound shows only competitive inhibition, that is the rate of hydrolysis of substrate is immediately slowed down but is not stopped and the enzyme is not inactivated.

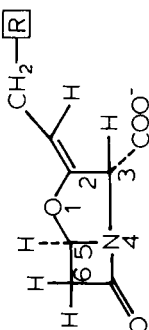
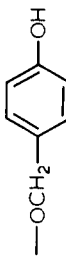
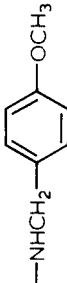
DERIVATIVES AND ANALOGUES OF CLAVULANIC ACID AS β -LACTAMASE INHIBITORS

The progressive inactivation of β -lactamases brought about by clavulanic acid is dependent on the presence of the β -lactam ring and on the structure of the side-chain in position 2. Reduction of the double bond in this side-chain gives dihydroclavulanic acid (BRL 18905) which is inactive as a β -lactamase inhibitor.

Substitution on the oxygen of the side-chain of clavulanic acid gives ethers and, as shown in *Table 2.3*, the *p*-hydroxybenzyl ether (BRL 23980) has similar activity to that of clavulanic acid. However, without pre-incubation BRL 23980 has a lower I_{50} value against staphylococcal β -lactamase, indicating greater competitive inhibitory activity, but the compound is also an inactivator of the enzyme. Activity against the cephalosporinase of *Ent. cloacae* P99 is not significantly altered by this modification.

Replacement of the side-chain hydroxyl of clavulanic acid by a substituted amino group gives compounds of greater activity against certain β -lactamases (*Table 2.3*). For example, the *p*-methoxybenzylamine (BRL 23654) shows improved activity against both staphylococcal and TEM β -lactamases with

Table 2.3 EXAMPLES OF CLAVULANIC ACID DERIVATIVES WITH β -LACTAMASE INHIBITORY ACTIVITY

Clavulanate derivative	R	<i>I</i> ₅₀ value ^a (μg/ml) against β-lactamase preparations from:				
		<i>Staphylococcus aureus</i> (Russell) (+)	<i>Escherichia coli</i> (JT4 R ⁺ TEM) (+)	<i>Enterobacter cloacae</i> P99 (+)		
						
Clavulanate	—OH	0.03	77	0.07	0.65	> 50
ether, BRL 23980		0.04	4	0.07	0.16	50
amine, BRL 23654		0.002	1.2	0.0014	0.018	20

Source: Cole and Reading (1982).

^a With (+) or without (−) 5 min pre-incubation between β -lactamase and inhibitor before adding nitrocefin substrate (250 $\mu\text{g/ml}$).

and without pre-incubation with the enzyme. This suggests that it initially acts as a good competitive inhibitor before reacting with the enzyme. Newall (1981) has also described certain clavulanic acid derivatives which have improved β -lactamase inhibitory properties. Various synthetic analogues of clavulanic acid prepared as mixtures of isomers at C-5 have been shown to have β -lactamase inhibitory activity. The structures and inhibition profiles of some of these compounds have been discussed (Cole, 1980).

MECHANISM OF INHIBITION OF β -LACTAMASE BY CLAVULANIC ACID

Clavulanic acid has been termed an active-site-directed inhibitor because it resembles a substrate molecule by fitting into the catalytic centre of the β -lactamase and reacting with the enzyme. However, whereas a substrate molecule would very rapidly leave the enzyme surface in the form of its degradation product, the clavulanic acid remains attached as a result of further chemical reactions.

Knowles and his research colleagues have investigated the kinetic and chemical aspects of the interaction of clavulanic acid with purified TEM-2 β -lactamase (Fisher *et al.*, 1980, and references therein). They found two forms of inactivated enzyme in the early stages of the reaction with clavulanic acid, namely a transiently inactivated form which regained enzyme activity in the absence of clavulanic acid and a permanently inactivated form which did not. On prolonged incubation, all of the enzyme was converted into the irreversibly inactivated form. During these reactions, some molecules of clavulanic acid are destroyed as though the compound was initially acting as a substrate.

Reading and Hepburn (1979) investigated the interaction of clavulanic acid with the staphylococcal β -lactamase and found that an inactive complex was rapidly formed, one molecule of clavulanic acid being required to inactivate each molecule of β -lactamase. A comparison of the interaction of clavulanic acid with various β -lactamases has been made by Reading and Farmer (1981). They found that both the TEM-2 β -lactamase from *E. coli* and the β -lactamase of *Klebsiella* rapidly formed ($T_1 < 1$ min) a transiently stable, inactive complex with clavulanic acid. Both enzymes were then converted more slowly to irreversibly inactivated complexes, the T_1 values being 15 min and 3 min, respectively.

In contrast, the β -lactamase of *Proteus mirabilis* and *Staph. aureus* rapidly formed moderately stable inactive complexes, the T_1 value for the former being 1 min and the latter, at a concentration of 2 $\mu\text{g/ml}$, being 2.5 min. The T_1 values for the decay of the latter complexes in the absence of clavulanic acid were 40 min and 160 min, respectively. The β -lactamase of *Ent. cloacae* needed a high concentration of clavulanic acid to inhibit it, 240 $\mu\text{g/ml}$ taking 2.5 min to form a moderately stable complex with a decay T_1 of 180 min.

The experiments described above were carried out using preparations of β -lactamase isolated from bacterial cells. The results illustrated in Figure 2.6 show that clavulanic acid can also inhibit the destruction of amoxycillin by whole washed cells of a strain of *E. coli* which produces the TEM-1 β -lactamase (Reading, 1981).

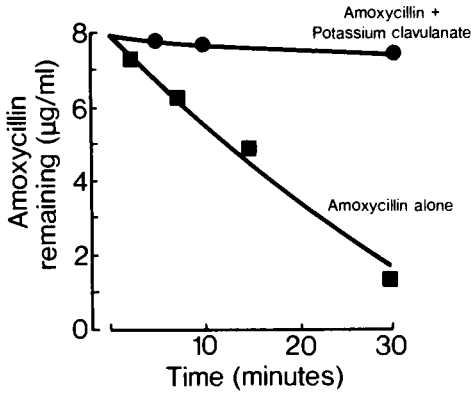


Figure 2.6 The protection of amoxycillin (8 µg/ml) by potassium clavulanate ($\equiv$ 4 µg/ml clavulanic acid) from degradation by whole cells (5×10^7 cells/ml) of *Escherichia coli* JT4 (TEM-1)

USE OF POTASSIUM CLAVULANATE TO PROTECT PENICILLINS FROM DESTRUCTION BY β -LACTAMASE

The ability of potassium clavulanate (equivalent to 2 µg/ml clavulanic acid) to inhibit the β -lactamase hydrolysis of low concentrations (8 µg/ml) of various penicillins is illustrated in *Figure 2.7* (Cole and Reading, 1982). The assays of residual substrate were carried out by HPLC methods. It can be seen that the action of the staphylococcal β -lactamase was arrested very quickly, there being no further hydrolysis of any of the penicillins. The extent of hydrolysis of penicillins which occurred before the enzyme was inactivated reflects a combination of the relative affinities of the penicillins for the β -lactamase and relative rates of hydrolysis of the substrates. In the absence of clavulanic acid, ticarcillin was the most slowly hydrolysed penicillin, the initial rate being 0.38 (µg/ml)/min at a substrate concentration of 8 µg/ml. The corresponding initial rates for the other penicillins were, in (µg/ml)/min: 6.7 for amoxycillin, 7.4 for ampicillin and 7.8 for piperacillin, the same enzyme concentrations being used for all reactions.

Similar results were obtained with the TEM β -lactamase (*Figure 2.8*), there

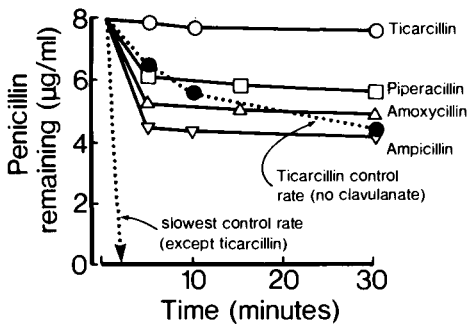


Figure 2.7 Inhibition of *Staphylococcus aureus* (Russell) β -lactamase hydrolysis of various penicillins by potassium clavulanate. Beta-lactamase + 8 µg/ml penicillin + potassium clavulanate ($\equiv$ 2 µg/ml clavulanic acid)

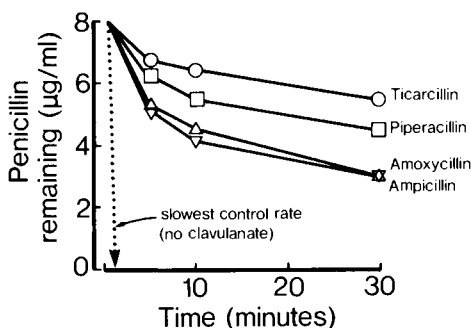


Figure 2.8 Inhibition of *Escherichia coli* JT4 R⁺ TEM-1 β -lactamase hydrolysis of various penicillins by potassium clavulanate. Beta-lactamase + 8 μ g/ml penicillin + potassium clavulanate ($\equiv$ 2 μ g/ml clavulanic acid)

being a rapid slowing down of the hydrolysis of all of the penicillins, but the reactions were not completely arrested within the time course of this experiment. The same dilutions of the enzyme preparation were used for all of the reactions. In the absence of clavulanic acid the initial rates of hydrolysis of 8 μ g/ml substrate ranged from 8 (μ g/ml)/min for ticarcillin to 37.4 (μ g/ml)/min for amoxycillin, the values for ampicillin and piperacillin being 30.6 and 32.6 (μ g/ml)/min, respectively. In spite of these high enzyme reaction rates, it can be calculated that the 2 μ g/ml clavulanic acid brought about greater than 99% inhibition of the rate of hydrolysis of all the penicillins in 5 min.

The protective effect of clavulanic acid is further illustrated by the results in Table 2.4. In these experiments (Reading, Farmer and Cole, 1983), various preparations of plasmid- and chromosomally-mediated β -lactamases were incubated with amoxycillin (16 μ g/ml) in the presence of potassium clavulanate ($\equiv$ 4 μ g/ml clavulanic acid) and the amoxycillin remaining at 30 min assayed by HPLC methods. The enzyme preparations were made by disrupting the bacterial cells with ultrasonics and removing cell wall fragments by centrifugation. The activities of the preparations against amoxycillin were very high, most of them giving a hydrolysis rate in excess of 8 (μ g/ml)/min, but in the presence of clavulanic acid substantial concentrations of amoxycillin, often in excess of 50%, were still present at 30 min. *Escherichia coli* JT410 produces a chromosomally mediated β -lactamase which is active against certain cephalosporins, but is not readily inhibited by clavulanic acid. However, amoxycillin has good stability to this type of β -lactamase, as indicated by its slow rate of hydrolysis which was less than 0.1 (μ g/ml)/min (Table 2.4).

POTENTIATION OF THE ANTIBACTERIAL ACTIVITY OF AMOXYCILLIN BY POTASSIUM CLAVULANATE

The reduction in the bactericidal activity of amoxycillin against resistant bacteria can be shown in many cases to be associated with the destruction of the compound by β -lactamase action. This is illustrated for a β -lactamase-producing strain of *Staph. aureus* (Russell) in Figure 2.9. The residual concentrations of amoxycillin are shown in parentheses and it can be seen that within

Table 2.4 INHIBITORY EFFECT OF POTASSIUM CLAVULANATE ON THE DESTRUCTION OF AMOXYCILLIN BY VARIOUS β -LACTAMASES

Source and type of β -lactamase preparation	Plasmid	Enzyme	Activity of β -lactamase preparation against amoxycillin ($\mu\text{g}/\text{ml}/\text{min}$)	Amoxycillin ($\mu\text{g}/\text{ml}$) remaining at 30 min in a mixture of amoxycillin (16 $\mu\text{g}/\text{ml}$), potassium clavulanate ($\equiv$ 4 $\mu\text{g}/\text{ml}$ clavulanic acid) and β -lactamase preparation
<i>Escherichia coli</i> JT4	R ⁺	TEM-1	>8	8.5
<i>E. coli</i> K12	RP1	TEM-2	>8	6.9
<i>E. coli</i> K12 J53/2	pR1010	SHV-1	>8	12.0
<i>E. coli</i> K12	R1818	OXA-2	2.7	11.2
<i>E. coli</i> K12 E5/2	pMG19	PSE-4	>8	8.3
<i>E. coli</i> JT410	—	—	<0.1	15.5
<i>Proteus mirabilis</i> C889	—	—	>8	6.4
<i>Klebsiella pneumoniae</i>	—	—	>8	10.2
ATCC 29665	—	—	>8	10.2
<i>Staphylococcus aureus</i> (Russell)	+ve	—	7.4	10.9

3 h the initial concentration of 2 µg/ml was reduced to an undetectable level (<0.1 µg/ml) and the culture started to grow. A very different situation was seen when potassium clavulanate was also present. Using a concentration of 1 µg/ml amoxycillin with potassium clavulanate at a concentration equivalent to 0.5 µg/ml clavulanic acid, there was an initial loss of amoxycillin, but thereafter the concentration remained between 0.5 and 0.6 µg/ml for at least 24 h and was associated with a bactericidal effect. The initial loss of amoxycillin probably occurs during the time it takes for the clavulanic acid to inactivate the β -lactamase.

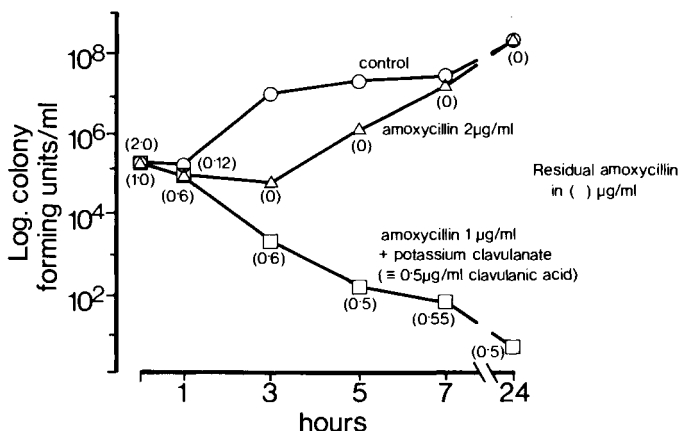
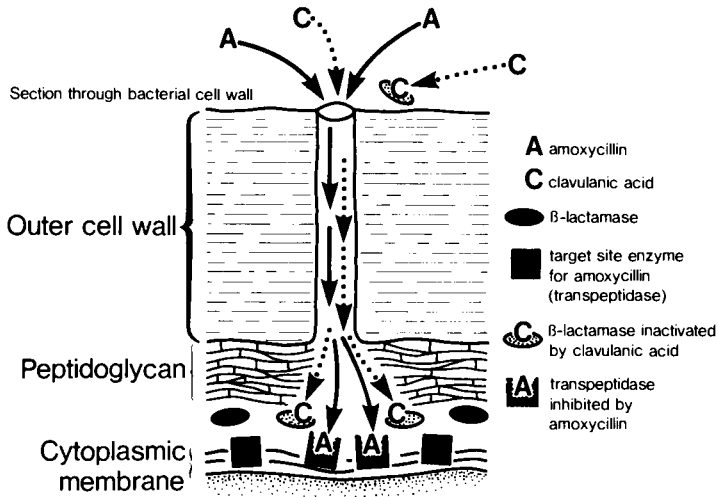


Figure 2.9 Effect of amoxycillin and amoxycillin + potassium clavulanate on the viability of a β -lactamase-positive culture of *Staphylococcus aureus* (Russell)

The mode of antibacterial action of amoxycillin in the presence of clavulanic acid (or more strictly, the clavulanate ion) against bacteria which owe their resistance to β -lactamase production, thus appears to depend on the prevention of the destruction of the amoxycillin. As β -lactamase molecules are sited in the cell wall in the same region as the penicillin-sensitive target enzymes, the amoxycillin concentration in this region could be reduced to the point where it was inadequate to inhibit these enzymes. In the presence of clavulanic acid, the concentration of amoxycillin is maintained at a level sufficient to inhibit the target enzymes. The way in which this is achieved is shown diagrammatically in Figure 2.10.

Many studies have been published describing the *in vitro* and *in vivo* potentiation of the antibacterial activity of penicillins and cephalosporins by salts of clavulanic acid against β -lactamase-producing strains of bacteria (Hunter *et al.*, 1980; Comber *et al.*, 1980; reviewed by Cole, 1981). The most widely studied combination is clavulanic acid plus amoxycillin, because clavulanic acid inhibits a wide range of the important β -lactamases which destroy amoxycillin and both compounds can be administered orally.

Boon *et al.* (1982) studied the fate and efficacy of 2:1 or 4:1 formulations of amoxycillin and clavulanic acid dosed orally to mice infected with various β -lactamase-producing, amoxycillin-resistant bacteria. The infections included *Staph. aureus* and *E. coli* in the peritoneum, a staphylococcal thigh



Clavulanic acid (as its clavulanate ion) reacts with β-lactamase molecules and thereby inhibits the destruction of amoxycillin. Amoxycillin molecules are then available to block the action of the transpeptidase involved in cell wall construction.

Figure 2.10 Mode of action of amoxycillin in the presence of clavulanic acid against a β-lactamase-producing bacterium

lesion, a *K. pneumoniae* pneumonia, a *Bacteroides fragilis* groin abscess and an *E. coli* pyelonephritis. Amoxycillin and clavulanic acid were found at significant concentrations at the various sites of infection; for example, peritoneal washings, infected tissue homogenates, pleural fluid and pus. Amoxycillin concentrations were higher in the presence of clavulanic acid than in its absence, as a result of inhibition of the β-lactamase at the site of infection. The protective effect of clavulanic acid on amoxycillin was further illustrated by the efficacy of the combination against the infections studied, most of which were resistant to amoxycillin alone.

BETA-LACTAMASES IN VETERINARY ISOLATES AND THEIR INHIBITION BY CLAVULANIC ACID

Few surveys on the types of β-lactamase present in ampicillin-resistant strains of bacteria from animal sources have been reported. Medeiros, Gillece and O'Brien (1981) examined 261 non-typhoidal ampicillin-resistant salmonellae, 114 of human origin and 146 from animals. Examination of crude sonic extracts of the bacterial cells by the isoelectric focusing method revealed known plasmid-mediated β-lactamases in all of the strains. The most prevalent type of enzyme was the TEM-1 ($n=203$) followed by OXA-2 ($n=50$), OXA-1 ($n=3$) and PSE1 ($n=2$). Three isolates each contained pairs of these enzymic types. The OXA-2 β-lactamase was found in 16 out of 52 animal isolates of *Salmonella typhimurium*, but only 4 of 38 human isolates. Hirsh, Assaf and Libal (1981) reported the presence of a plasmid-mediated TEM β-lactamase in an ampicillin-resistant strain of *Haemophilus pleuropneumoniae* isolated from swine.

In our laboratories we have investigated the types of β -lactamase present in a small collection of amoxycillin-resistant bacteria obtained from field trials in the UK and on the continent. The bacteria (Table 2.5) were grown for 7.5 h in a tryptone-based medium in flasks shaken on a rotary shaker at 37°C. To induce β -lactamase formation, methicillin (1.0 $\mu\text{g/ml}$) or amoxycillin (0.5 $\mu\text{g/ml}$) was added to the staphylococcal cultures, amoxycillin (20 $\mu\text{g/ml}$) to the *Enterobacter* culture and amoxycillin (500 $\mu\text{g/ml}$) to the *Proteus* FR2 culture. After collecting the cells by centrifugation, they were disrupted by ultrasonics and the debris removed by centrifugation. The supernatants were examined for β -lactamase activity by measuring the rate of hydrolysis of 50 or 500 $\mu\text{g/ml}$ amoxycillin or 500 $\mu\text{g/ml}$ cephaloridine in a spectrophotometer. The enzymes were characterized by isoelectric focusing on LKB PAG plates in the pH range 3.5–9.5 using the LKB Multiphor apparatus and nitrocefin substrate to stain the bands.

Table 2.5 BETA-LACTAMASES FOUND IN AMOXYCILLIN-RESISTANT STRAINS OF BACTERIA OF VETERINARY ORIGIN

Strain of bacterium		Rate of hydrolysis of amoxycillin by sonicated cells from a 7 h culture ($\mu\text{g/ml}$)/min	Types of β -lactamase based on isoelectric focusing (pI values in parentheses) and substrate hydrolysis	
<i>Escherichia coli</i>	DU2	190	TEM-1	(5.5)
	DU24	300	TEM-1	(5.5)
	CE2	270	TEM-1	(5.5)
	FR16	150	TEM-1	(5.5)
	E127	0.93 (100) ^a	Like chromosomal cephalosporinase of <i>E. coli</i> JT410	(9.1)
	E135	2.4 (350)	Cephalosporinase	(9.25)
	E136	450 (570)	TEM plus cephalosporinase	(5.5 and 9.25)
<i>Klebsiella</i> spp.	DU1	470	Like chromosomal β -lactamase of <i>K. aerogenes</i> E70	(7.0)
	CE1	40	Slightly different from SHV-1	(7.5)
	CE6	130	TEM-1	(5.5)
<i>Proteus</i> spp.	DU4	250	TEM-2	(5.65)
	DU27	270	TEM-2	(5.65)
	FR2	2.3 (13.5)	Like cephalosporinase of <i>P. vulgaris</i>	(9.3)
			New type—penicillinase	
<i>Citrobacter</i> sp.	DU1	37	Cephalosporinase different from <i>E. cloacae</i> P99	(6.5)
<i>Enterobacter</i> sp. (amoxycillin induced)	DU10	<0.5 (4.4)		(7.7)
<i>Staphylococcus</i> spp. (methicillin induced) (amoxycillin induced)	DU72	240	Staphylococcal	(—)
		15		
	FR9	150	Staphylococcal	(—)
		2.7		

^a β -Lactamase activity measured using cephaloridine as substrate.

The results in Table 2.5 reveal that many of the types of β -lactamase were similar to those which have been observed in cultures of human origin. The plasmid-mediated TEM-1 β -lactamase was present in all of the strains of *E. coli* which rapidly hydrolysed amoxycillin, whereas the others produced a cephalosporinase which had high activity against cephaloridine. One culture of *E. coli* (E136) produced the two types of enzyme and E127 produced a cephalosporinase resembling the one present in the human isolate of *E. coli* JT410 (Reading and Cole, 1977). One of the *Klebsiella* cultures produced a β -lactamase which behaved the same as that in a human isolate of *K. aerogenes* (E70) (Reading and Farmer, 1981), another contained the TEM-1 β -lactamase, while the third produced an enzyme resembling the plasmid-mediated SHV-1 enzyme. Two *Proteus* cultures produced the plasmid-mediated TEM-1 β -lactamase, while the third behaved like a typical cephalosporinase-producing culture of *P. vulgaris*. The *Citrobacter* culture produced a new type of penicillinase, while the *Enterobacter* culture did not hydrolyse amoxycillin at a significant rate. The staphylococcal β -lactamase was more readily induced by the β -lactamase stable penicillin, methicillin, than by amoxycillin; this enzyme cannot readily be analysed by isoelectric focusing.

Table 2.6 INHIBITION OF β -LACTAMASES IN VETERINARY ISOLATES BY CLAVULANIC ACID AND POTENTIATION OF ACTIVITY OF AMOXYCILLIN

Beta-lactamase-producing veterinary isolates of bacteria		Inhibition of β -lactamase by potassium clavulanate, I_{50} value ($\mu\text{g/ml}$) ^a		Minimum inhibitory concentration ($\mu\text{g/ml}$) for amoxycillin in agar ^b	
				Amoxycillin alone	Amoxycillin in presence of potassium clavulanate (4:1)
<i>Escherichia coli</i>	DU2	—	(TEM-1)	> 80	20
	DU24	0.044	(TEM-1)	> 80	20
	CE2	—	(TEM-1)	> 1280	20
	FR16	—	(TEM-1)	> 80	10
	E127	154		320	80
	E135	180		640	80
	E136	31		1280	80
<i>Klebsiella</i> spp.	DU1	0.021		80	1.25
	CE1	0.029		80	5
	CE6	0.039	(TEM-1)	> 1280	20
<i>Proteus</i> spp.	DU4	—	(TEM-2)	> 80	5
	DU27	0.041	(TEM-2)	> 80	5
	FR2	0.012		80	2.5
<i>Citrobacter</i> spp.	DU1	0.0035		40	2.5
<i>Enterobacter</i> spp.	DU10	74		> 80	80
<i>Staphylococcus</i> spp.	DU72	0.11		5	0.62
	FR9	0.10		10	0.62

^a The I_{50} value was the concentration giving 50% inhibition of enzyme activity after 5 min contact between enzyme and potassium clavulanate. The enzyme dilutions were adjusted so as to give a rate of hydrolysis of nitrocefin (250 $\mu\text{g/ml}$) of about 8 ($\mu\text{g/ml}$)/min for all enzymes except those of E127, E135, E136 and DU10, where cephaloridine (250 $\mu\text{g/ml}$) was used and the rate was about 14 ($\mu\text{g/ml}$)/min.

^b Amoxycillin concentration expressed in terms of pure free acid. Amoxycillin trihydrate and potassium clavulanate used at a ratio of 4:1 on a pure free acid basis.

Clavulanic acid was found to readily inhibit all of the amoxycillin hydrolysing β -lactamases, but not the cephalosporinases produced by *E. coli* strains E127, E135, E136 and *Enterobacter* DU10 (Table 2.6). These results are similar to those observed in our laboratories for β -lactamases from human isolates.

Antibacterial tests revealed marked potentiation of the activity of amoxycillin by clavulanic acid against the cultures for which evidence of β -lactamase inhibition was obtained. The culture of *E. coli* E127 and E135 and *Enterobacter* DU10 produced a β -lactamase with low activity against amoxycillin. This activity may have been sufficient to explain the resistance to amoxycillin, but the cultures may also have been intrinsically resistant to the compound. The addition of clavulanic acid appeared to reduce the MIC value for amoxycillin, but in view of its poor inhibitory action against the β -lactamase of these organisms the effect is almost certainly due to the antibacterial activity of the clavulanic acid which was present at 20 $\mu\text{g/ml}$ (i.e. one-quarter of the amoxycillin concentration). The *E. coli* E136 produced both TEM and cephalosporinase enzymes and was highly resistant. The fall in MIC value when clavulanic acid was added reflects the inhibition of the TEM enzyme, but the culture still remained relatively resistant because of the second β -lactamase or intrinsic resistance.

Conclusions

Very many compounds have been shown to have β -lactamase inhibitory activity. The most interesting have a β -lactam ring fused with another ring system as in penams (e.g. cloxacillin, penicillanic acid sulphone and halopenicillanic acids), cephems, 7-methoxy cepheems and oxacephems (e.g. cefoxazole, cefoxitin and moxalactam), carbapenems (e.g. olivamic acids and carpenimycins) and clavams (e.g. clavulanic acid). Side-chain substituents in these molecules clearly affect their activity, breadth of spectrum and mechanism of inhibition; for example, whether they are competitive inhibitors, enzyme inactivators or a combination of the two.

All of the above compounds have been shown to potentiate the activity of labile β -lactam antibiotics against certain β -lactamase-producing bacteria. However, the most extensively studied, *in vitro* and *in vivo*, are salts and esters of sulbactam and salts of clavulanic acid. These compounds have been compared for their ability to potentiate ampicillin (Wise, Andrews and Bedford, 1980; Hunter and Webb, 1980), piperacillin (Neu and Fu, 1980), azlocillin (Calderwood *et al.*, 1982) mecillinam (Neu, 1982) and cefoperazone (Fu and Neu, 1981; Crosby and Gump, 1982).

The degree of potentiation of the antibacterial activity of a penicillin or cephalosporin by a β -lactamase inhibitor will depend on many factors, such as the extent to which β -lactamase is involved in the resistance, the ease with which clavulanic acid inhibits the β -lactamase and the intrinsic sensitivity of the bacterium to the antibiotics being protected. The substantial potentiation of the activity of amoxycillin by clavulanic acid is now well documented in reports of *in vitro* and *in vivo* bacteriological experiments (Cole, 1981, review). Clavulanic acid is particularly suitable for use with amoxycillin because it inhibits a wide range of the β -lactamases which destroy this penicillin and the

two compounds can be co-administered orally. Mizen *et al.* (1981) described the pharmacokinetics and distribution of amoxycillin and clavulanic acid in healthy animals, and Boon *et al.* (1982) the distribution and efficacy in infected animals. Staniforth, Lillystone and Jackson (1982) have reported the bioavailability and tolerance in humans.

Encouraging results have been obtained in clinical trials in which the two compounds were co-administered to treat a variety of human infections (for review, see Cole, 1981). A formulation of amoxycillin trihydrate with potassium clavulanate is now available in the UK under the trade name Augmentin (Beecham Pharmaceuticals). A veterinary formulation with the trade mark Synulox (Beecham Pharmaceuticals) has recently been introduced for treating infections in small animals. Laboratory and field trial results on the latter were described at the British Veterinary Congress, in 1982 (Bywater, 1982). Experimentally induced skin lesions in the dog, caused by staphylococci, were shown to resolve more rapidly when treated with Synulox than with amoxycillin. In field trials with Synulox, higher success rates were recorded for skin conditions and enteritis than obtained with amoxycillin and a high success rate was obtained for the treatment of amoxycillin-resistant urinary tract infections.

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ACTION OF NITRIFICATION INHIBITORS

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Nitrification and nitrogen cycling in soils

Major components of the nitrogen cycle in agricultural soils are summarized in *Figure 3.1*. Ammonium nitrogen may be added to soils directly, as ammonium-containing fertilizers, or indirectly in compounds containing organic nitrogen such as plant residues or animal slurries. Soil microorganisms convert organic nitrogen to ammonium nitrogen by ammonification (oxidative, reductive or direct deamination). In most aerobic soils, ammonium is then converted to nitrate by nitrifying bacteria at rates dependent on soil pH, temperature, aeration and ammonium concentration.

The process of nitrification involves two stages: oxidation of ammonium to nitrite, and oxidation of nitrite to nitrate. Each stage is undertaken by a distinct group of Gram-negative chemolithoautotrophic bacteria of the

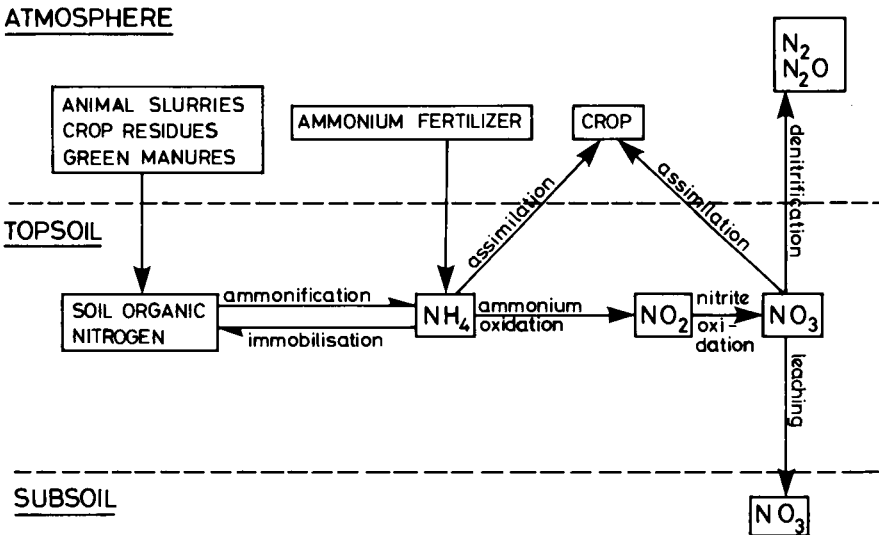


Figure 3.1 Nitrogen transformations in agricultural soils

family Nitrobacteriaceae. Ammonium-oxidizing bacteria include *Nitrosomonas* and *Nitrosolobus*, and nitrite-oxidizing bacteria include *Nitrobacter* and *Nitrocystis*. The bacteria utilize ammonium or nitrite as electron donors, and free energy changes (ΔG°) for ammonium or nitrite in aqueous solution at 1 mol/kg activity are +436 and +163 kJ/reaction, respectively (Thauer, Jungermann and Decker, 1977). Molar growth yields are approximately 0.7 g dry wt *Nitrosomonas* cell/mol ammonium oxidized and 0.3 g dry wt *Nitrobacter* cell/mol nitrite oxidized (Knowles, Downing and Barrett, 1965). In soils, ammonium and nitrite oxidation usually occur concurrently, but the latter is more rapid, so nitrite rarely accumulates except in high pH soils where the elevated pH inhibits nitrite oxidizers much more than ammonium oxidizers.

Most crops can assimilate inorganic nitrogen as either ammonium or nitrate. However, the nitrate ion is mobile in soils and may be leached by rain or irrigation water to below the maximum rooting depth, and thus be unavailable to the crop, whereas ammonium is relatively immobile in most soils. Also, in saturated soils anaerobic microsites may develop and in such conditions nitrate (but not ammonium) may be used by certain bacteria as a terminal electron acceptor in place of oxygen. This process is called denitrification, and in it nitrate is reduced to gaseous nitrogen oxides and nitrogen gas which volatilize from the soil. By inhibiting nitrification, inorganic nitrogen can be maintained in the topsoil as ammonium and, by preventing leaching or denitrification losses, crop recovery of nitrogen may be enhanced.

However, when a crop's demand for nitrogen is high but the risk of leaching/denitrification losses is low (usually from late spring onwards in the UK), it may be desirable for nitrification to proceed rapidly. There is, first, evidence which suggests that most plants require a mixture of both ammonium and nitrate for optimum growth and yield (Haynes and Goh, 1978). If nitrification is completely inhibited, only ammonium nitrogen will be present. Secondly, soil microorganisms may assimilate ammonium and immobilize it as organic biomass nitrogen more rapidly than nitrate, because soil microorganisms generally assimilate ammonium in preference to nitrate (Jansson, Hallam and Bartholomew, 1955). A (perhaps extreme) reason why the inhibition of nitrification must be temporary or partial is that denitrification of nitrate is the main process by which nitrogen is returned to the atmosphere. The amounts of nitrogen returned to the atmosphere from land and oceans annually as nitrogen gas plus nitrous oxide are small (3.5×10^2 Tg/year) compared with the total amount of nitrogen plus nitrous oxide in the atmosphere (3.9×10^9 Tg N) (Söderlund and Svensson, 1976), but the process is an essential part of the nitrogen cycle.

Nitrification inhibitors

Useful nitrification inhibitors should inhibit either both stages of nitrification or only ammonium oxidation, the first stage. If only the second stage, nitrite oxidation, is inhibited then nitrite would accumulate and the use of such an inhibitor would be dangerous, because the nitrite ion is phytotoxic. In practice, nitrite oxidizers are generally much less sensitive to most inhibitors than ammonium oxidizers, so the first stage is preferentially inhibited (Figure 3.2).

Many diverse substances inhibit nitrification (Bundy and Bremner, 1973;

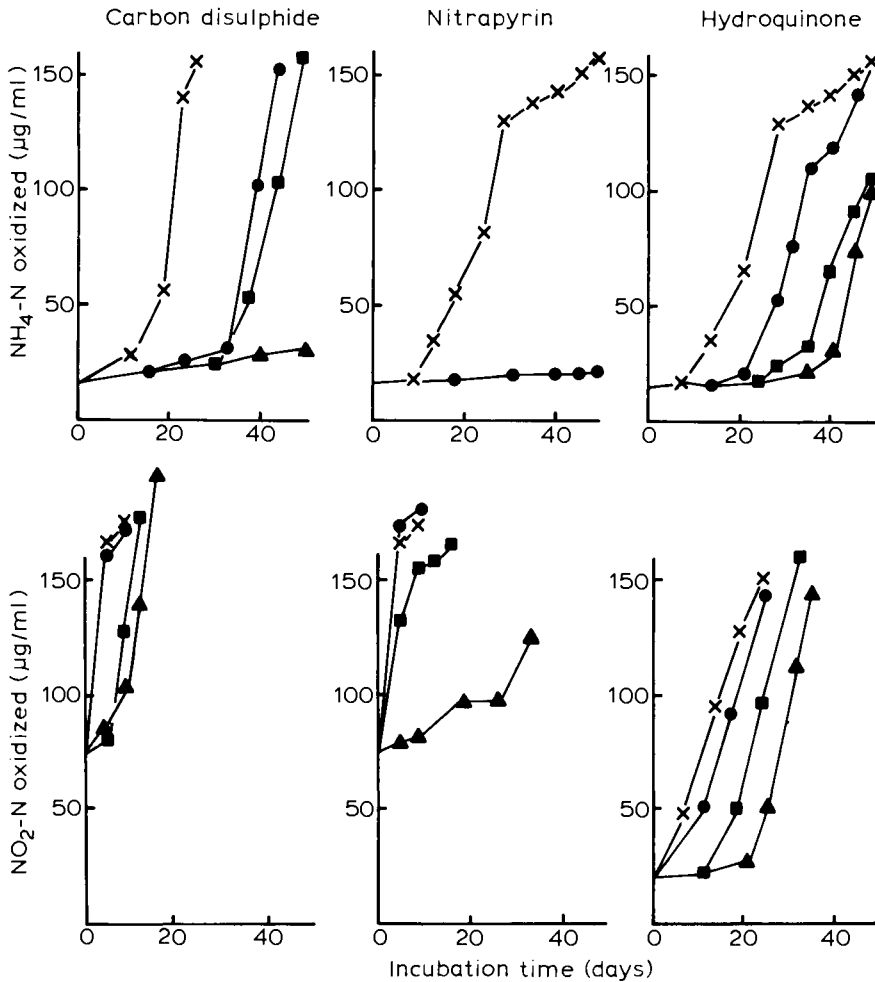
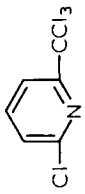
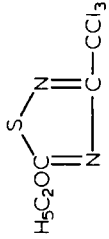
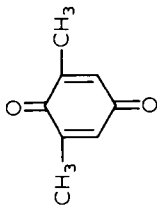
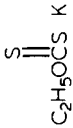
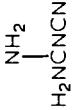


Figure 3.2 Effect of nitrification inhibitors on ammonium or nitrite oxidation in liquid culture: —x—, —●—, —■—, —▲—; 0, 1, 10 and 100 µg inhibitor per ml medium, respectively

Sahrawat, 1980). Compounds tested in field and laboratory experiments and discussed later are listed in *Table 3.1*; of these nitrapyrin, etridiazole and dicyandiamide are commercially available. Some mechanisms for their inhibitory properties are reviewed by Hauck (1980). Quinones and substituted quinones can retard the rate of urea hydrolysis to ammonium as well as being nitrification inhibitors. They may have a role in agricultural practice, primarily as urease inhibitors. Potassium ethyl xanthate *per se* is not a nitrification inhibitor but, after addition to soil, it slowly hydrolyses releasing carbon disulphide which is a potent nitrification inhibitor at low concentrations (Powlson and Jenkinson, 1971).

Table 3.1 NITRIFICATION INHIBITORS TESTED

<i>Inhibitor</i>	<i>Chemical name</i>	<i>Formula</i>	<i>Trade name</i>	<i>Source</i>	<i>Reference</i>
Nitrapyrin	2-Chloro-6-(trichloromethyl)pyridine		N-Serve 24E	Dow Chemical Co., King's Lynn, Norfolk, UK	Goring (1962)
Etridiazole	5-Ethoxy-3-trichloromethyl-1,2,4-thiadiazole		Dwell 4L	Midox Ltd, Oakham, Leicestershire, UK	Sommer (1972)
DMBQ	2,6-Dimethylbenzoquinone			Aldrich Chemical Co., Milwaukee, USA	Bundy and Bremner (1974)
KEIX	Potassium ethyl xanthate			BDH Ltd, Enfield, Middlesex, UK	Ashworth, Rodgers and Briggs (1979)
Dicyandiamide	Cyanoguanidine		Didin	SKW Ag., Munich, W. Germany	Reddy (1964)

Bacteriostatic and bactericidal action of inhibitors

Ammonium oxidation in aqueous media or soils usually resumes after inactivation of an inhibitor. Inhibitors may be bactericidal and kill ammonium oxidizers or they may be bacteriostatic agents and merely prevent ammonium oxidation. Laboratory experiments with *Nitrosolobus* in liquid culture indicate that most of the inhibitors tested were bacteriostatic (Table 3.2). The viability (determined by Most Probable Number counts) of early stationary phase *Nitrosomonas* cells after 48 h exposure to inhibitors was significantly decreased only by 100 µg nitrapyrin per ml medium and 1 or 10 µg etridiazole. Other inhibitors were not bactericidal, even at concentrations much higher than required for effective inhibition.

Table 3.2 EFFECT OF INHIBITORS ON VIABILITY OF *Nitrosolobus*

Inhibitor	Concentration (µg/ml)	Percentage viable cells after 48 h exposure to inhibitor	Concentration needed to inhibit nitrification in soil (µg/g)
Nitrapyrin	1	151	0.5-2
	10	93	
	100	0 ^a	
Etridiazole	1	8 ^a	0.5-2
	10	0 ^a	
2,6-Dimethyl- benzoquinone	100	31	5-10
Dicyandiamide	100	100	10-20
Potassium ethyl xanthate	100	33	10-20

^a Significant bactericidal effect ($p=0.05$) compared with controls incubated 48 h without inhibitor.

The two potentially bactericidal inhibitors, nitrapyrin and etridiazole, were further tested by amending soil in pots containing *Nitrosomonas* with 0.5 µg inhibitor and 200 µg ammonium nitrogen per g soil, followed by incubation at 20°C. Soils were sampled at intervals to determine ammonium, nitrate and *Nitrosomonas* numbers. In soil without an inhibitor, nitrification was measurable 24 h after the start of incubation (Figure 3.3). In inhibitor-treated soils, little nitrification was measured until approximately 25 days after the start of incubation. When nitrification eventually started, the rate was much slower in the etridiazole-treated soil than in the nitrapyrin-treated soil. Ammonium oxidizer numbers fell after addition of either nitrapyrin or etridiazole, especially in the etridiazole-treated soil. After 20 days, numbers slowly increased as nitrification started. This indicates that, in the soil used, low concentrations of either nitrapyrin or etridiazole were bactericidal. Both compounds were more bactericidal in soil than in aqueous culture, possibly because soil particles provided a surface for interaction of bacteria and inhibitor.

The inhibitors nitrapyrin or etridiazole (1.5 kg/ha) were injected with aqueous urea (375 kg N/ha) under a ryegrass ley at Rothamsted in November 1980 or March 1981, using a tractor-mounted injection rig. At intervals after injection, cross-sections of injected bands were taken with a nail-board and analysed for ammonium, nitrate and inhibitor concentrations and ammonium oxidizer numbers—see Ashworth and Flint (1974) for details of the

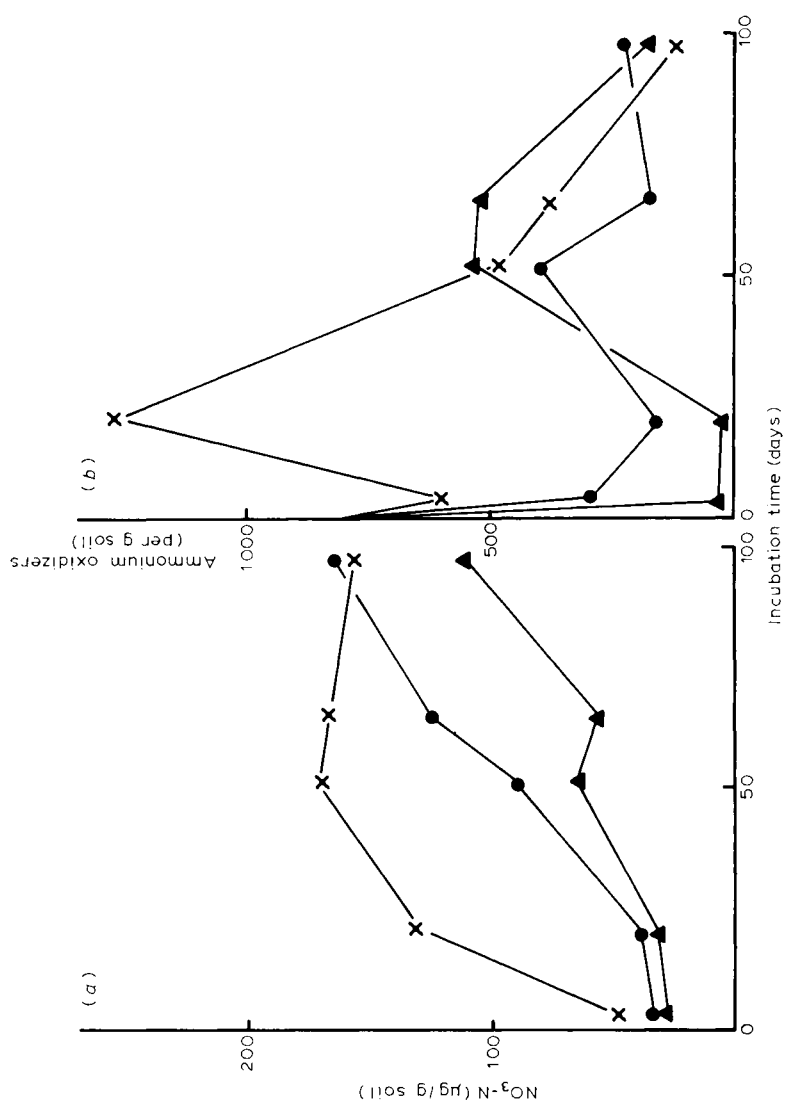


Figure 3.3 Nitrate concentrations (a), and numbers of ammonium-oxidizing bacteria (b), in loamy sand amended with 200 µg ammonium-nitrogen per g soil and incubated at 20°C: — x —, no inhibitor; — ● —, 0.5 µg nitrapyrin per g soil; — ▲ —, 0.5 µg etridiazole (after Rodgers and Ashworth, 1982)

Table 3.3 AMMONIUM AND NITRATE-NITROGEN, AMMONIUM-OXIDIZING BACTERIA AND INHIBITOR LEVELS IN SOIL INJECTED WITH 375 kg/ha UREA-NITROGEN IN NOVEMBER 1980. ALL VALUES IN kg/ha TO A DEPTH OF 20 cm. SAMPLED 42 DAYS AFTER INJECTION

<i>Treatment</i>	<i>NH₄-N</i>	<i>NO₃-N</i>	<i>Ammonium oxidizers</i>	<i>Inhibitor</i>
Urea only	320	65	0.0106	—
Urea + 1.5 kg/ha etridiazole	440	10	0.0118	0.5
Urea + 1.5 kg/ha nitrapyrin	390	25	0.0093	0.3

technique. Results (*Table 3.3*) from samples taken 6 weeks after November injection show that both inhibitors inhibited nitrification, but had little effect on numbers of viable ammonium oxidizers. Samples taken 5 days after injection in March also show that etridiazole had little effect on ammonium oxidizer numbers (*Table 3.4*). The 5-day interval between injection and sampling was long enough for the inhibitor to exert any bactericidal effect, but too short for any recovery from inhibition and for cell numbers to increase.

The laboratory experiments indicated that most of the inhibitors tested were not bactericidal, even at high concentrations. Nitrapyrin and etridiazole, at realistic concentrations, did exhibit some, but not 100%, bactericidal action in laboratory-incubated soils. In the field, neither nitrapyrin nor etridiazole were bactericidal.

Table 3.4 AS FOR *Table 3.3*, EXCEPT SAMPLES TAKEN 5 DAYS AFTER INJECTION IN MARCH 1981

<i>Treatment</i>	<i>NH₄-N</i>	<i>NO₃-N</i>	<i>Ammonium oxidizers</i>	<i>Inhibitor</i>
Urea only	374	19	0.0081	—
Urea + 1.5 kg/ha etridiazole	119	12	0.0092	0.2

Recovery from inhibition

Temporary inhibition of nitrification may be desirable, particularly when there is a high risk of loss of nitrified nitrogen, but, for reasons outlined previously, permanent inhibition is not advisable. Inhibitors added to soils or aqueous media gradually become inactive due to decomposition or volatilization. Provided not all the ammonium oxidizers are killed by an inhibitor, ammonium oxidation should resume after inhibitor inactivation. The time required for ammonium oxidation to resume in a nitrapyrin-treated elective culture of ammonium oxidizers from a pasture soil is shown in *Figure 3.4*. Addition of nitrapyrin to cultures which had resumed ammonium oxidation again inhibited the reaction. Other laboratory experiments with nitrapyrin (Laskowski and Bidlack, 1977; Meikle and Griffith, 1981) also showed that the inhibitor (2–10 µg/ml) did not kill all the ammonium-oxidizing bacteria. Belser and Schmidt (1981) observed considerable differences in sensitivity of ammonium oxidizers to nitrapyrin and that the degree of inhibition depended on the strain, rather than the genus, of ammonium oxidizer.

A simple model using growth rates, yields and saturation constants deter-

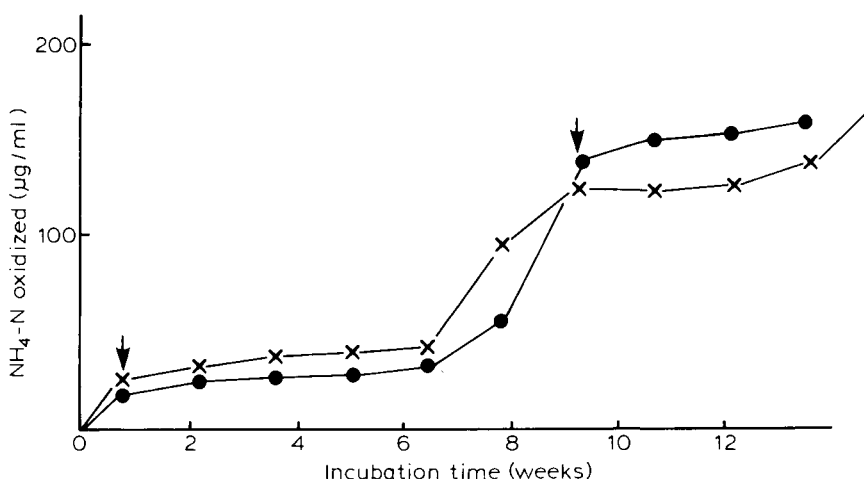


Figure 3.4 Recovery of ammonium oxidation in *Nitrosomonas* — x —, or *Nitrosolobus* — ● —, culture amended with 0.5 µg/ml nitrapyrin, at times arrowed (after Rodgers, Ashworth and Walker, 1980)

mined for *Nitrosomonas* by Knowles, Downing and Barrett (1965) demonstrates how the time required for oxidation of a given quantity of ammonium, after an inhibitor is inactivated, could depend on the numbers of bacteria surviving inhibitor treatment.

The model assumes exponential growth of ammonium oxidizers:

$$X_t = X_0 e^{\mu t}$$

where X_0 is the population of ammonium oxidizers surviving inhibitor treatment (µg cells/g soil); X_t is the final population of ammonium oxidizers after oxidation of all ammonium substrate (µg cells/g soil); t is the time for oxidation of all ammonium substrate (days); and μ is the specific growth rate (per day):

$$\mu = \frac{\mu_{\max} s}{K_s + s} \quad (\text{Monod, 1949})$$

where $\mu_{\max}$ is the maximum specific growth rate (per day) = 0.29 at 10°C; s is the ammonium concentration (µg NH₄-N per g soil); and K_s is the saturation constant (µg NH₄-N per g soil) = 0.22 at 10°C.

Yield of cells is related to the amount of ammonium-nitrogen oxidized by

$$\frac{X_t - X_0}{Y} + N_t = N_0$$

where N_0 is the ammonium concentration at time $t=0$ (µg NH₄-N per g soil); N_t is the ammonium concentration at time t (µg NH₄-N per g soil); and Y is the observed cell yield (µg cells/µg NH₄-N oxidized) = 0.05. Substituting for X_t :

$$\frac{X_0 e^{\mu t} - X_0}{Y} + N_t = N_0$$

If a soil contains $0.2 \mu\text{g}$ *Nitrosomonas* cells/g, $300 \mu\text{g}$ ammonium-nitrogen per g soil is added with an inhibitor, and nitrification starts at time $t=0$ after inactivation of the inhibitor, then the time required for oxidation of all the ammonium will depend on the number of ammonium oxidizers surviving the inhibitor treatment. Figure 3.5 shows how the time required for oxidation of $300 \mu\text{g}$ ammonium-nitrogen per g soil increases as the number of ammonium oxidizers that survive inhibitor treatment decreases. Death of 99% of the population will double the time required to oxidize the ammonium when nitrification resumes after inhibitor inactivation, compared with a population that is fully viable.

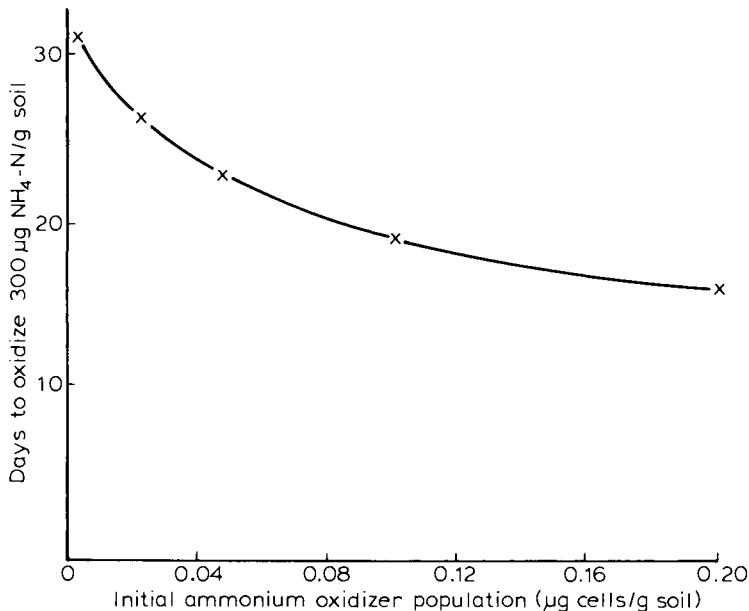


Figure 3.5 Effect of ammonium oxidizer population ($\mu\text{g cells/g soil}$) surviving inhibitor treatment, and present at time $t=0$ days when nitrification resumes, on the time for complete oxidation of $300 \mu\text{g}$ ammonium-nitrogen per g soil

Practical use of inhibitors

Cost-effective use of inhibitors in the UK may, in future, be a routine procedure, even in the absence of legislation concerned with nitrate pollution of groundwaters. The increased usage of urea fertilizer in the UK may justify its application with nitrification inhibitors because, unlike ammonium nitrate, none of the urea-nitrogen is initially in the nitrate form when applied to soils. Also, there is a trend for nitrogen applications for various crops to be given earlier. Soil conditions at the time of early nitrogen applications (i.e. winter and early spring) may be conducive to considerable losses of nitrate by leaching or denitrification. Amounts of nitrogen annually are increasing and, despite improved husbandry, so may nitrogen losses. In England and Wales, 1.4×10^6 t of manufactured fertilizer nitrogen were applied in 1981 (Church,

1982) at a cost of approximately £450 × 10⁶. Crop recovery of applied nitrogen fertilizer varies greatly, with values usually ranging from 20% to 80%. Although some fertilizer nitrogen is immobilized as organic nitrogen by soil microflora, much is undoubtedly lost by leaching and/or denitrification. Many data indicate that retention of nitrogen in agricultural systems may be very small (Greenwood, 1982).

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NITRIFICATION INHIBITION PROPERTIES OF ETRIDIAZOL

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Introduction

Many pesticides are known to affect the activity of soil microorganisms, including nitrification. As with fungicides, herbicides and insecticides, most of the compounds which inhibit nitrification are organic chemicals. At least 100–200 compounds are known to possess nitrification inhibition properties (Hanson, 1978). Although such compounds have been known in the literature for about 60 years under the designation 'nitrification inhibitors', only a few have received serious commercial development.

Etridiazol [5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole] was introduced in 1966 for seed and soil treatment against a number of diseases (Al-Bedawi and Sinclair, 1969). It was found to be especially effective for controlling pre- and post-emergence damping-off caused by *Pythium* and *Phytophthora*. Such uses have since been widely commercialized and the compound is a world-known soil and seed fungicide under various trademarks. In the UK and some of the Western European countries, the chemical is commercially known as Aaterra,* whereas Terrazole† has been a more common designation in the other parts of the world. In Japan, the product is marketed under the Pansoil‡ trademark.

The nitrification inhibition properties of etridiazol were first described by Sommer (1972). While studying several nitrification inhibitors of US and Japanese origin in culture of nitrifiers and in soil, he concluded that etridiazol was the most effective 'ammonium nitrifide' of the seven tested. He defined nitrificides as the chemicals that slow down or completely block the microbial oxidation of ammonium to nitrate in the soil, and ammonium nitrificides as those that affect *Nitrosomonas* without affecting *Nitrobacter*. Nowadays these compounds are known in the literature as nitrification inhibitors, and are defined as those that prevent or retard the first step in the process of nitrification of ammoniacal nitrogen by the *Nitrosomonas* bacteria.

Since the early research by Sommer, these chemicals have received considerable attention and have been extensively studied. They are considered as new

* Aaterra is the trademark of Aagrunol, Groningen, Holland.

† Terrazole is the trademark of Olin Corporation, Stamford, Connecticut, USA.

‡ Pansoil is the trademark of Sankyo Co. Ltd, Tokyo, Japan.

tools in the management of nitrogen in the soil. Of the many compounds known to have such a property, nitrapyrin (N-Serve*) and etridiazol (DWELL†) have been studied the most and are now commercialized for use in the USA and some of the countries in Europe and other parts of the world. In this chapter, etridiazol, Terrazole and DWELL are used interchangeably.

Effect of etridiazol on the soil nitrification process

Nitrogen for use in the biological processes of growing plants can be obtained from a number of different sources. These sources include commercial fertilizers, plant residues, fixation by legumes, animal manures and environmental contaminants in rainfall. Organic nitrogen is converted to inorganic ammonium nitrogen (NH_4^+) by the process of biological mineralization. The ammonium nitrogen is subsequently converted to the nitrate form (NO_3^-) by nitrification (Figure 4.1). Normally, these processes of natural chemical change

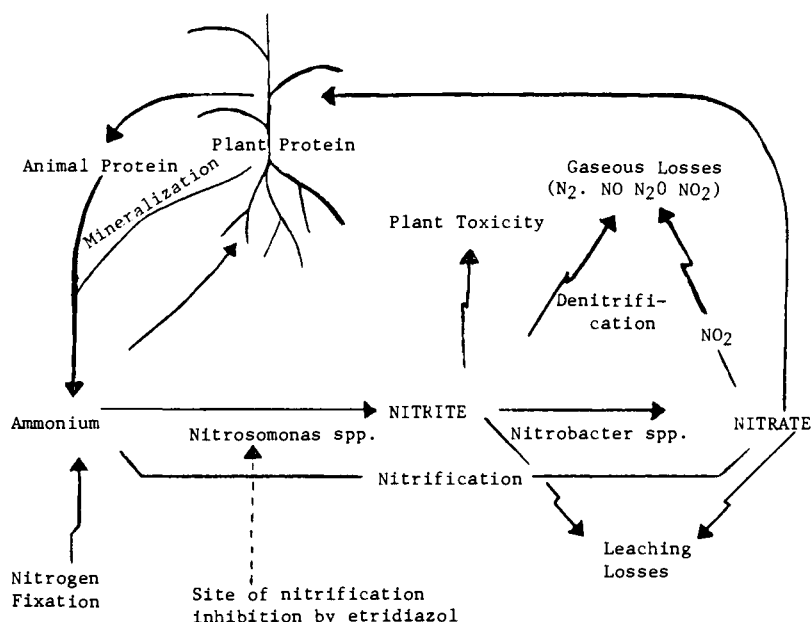


Figure 4.1 Schematic presentation of the nitrogen cycle (from Huber *et al.*, 1977, by courtesy of Dr D.M. Huber)

are uncontrolled by man, but in certain cases they can deprive crops of significant amounts of the nitrogen which has been applied.

Most nitrogen in the soil, whether from applied fertilizer, animal or crop residue, undergo mineralization and are converted to ammonium (Figure 4.1). Through various chemical or biological processes, these sources of ammonia are converted to the ammonium (NH_4^+) form of nitrogen. In this

* N-Serve is the trademark of the Dow Chemical Company, Midland, Michigan, USA.

† DWELL is the trademark of Olin Corporation, Stamford, Connecticut, USA.

positively charged form, the nitrogen is not susceptible to movement in the soil because it is adsorbed to the negatively charged soil particles, yet remains available for usage by plants.

Conversion of the stable ammonium form is continued in a two-step bacterial process known as nitrification. First, ammonium is converted to nitrite (NO_2^-) form of nitrogen as a result of the activity of *Nitrosomonas* spp. bacteria. This nitrite form is unstable and subject to leaching and gaseous loss. In addition, plants are unable to utilize the nitrite and are adversely affected by its presence.

In the second step of nitrification, the nitrite nitrogen is oxidized to the nitrate (NO_3^-) form of nitrogen by the action of *Nitrobacter* spp. bacteria. While plants are able to use nitrate as a source of nitrogen, it is an unreliable source. It can be rapidly lost through the leaching action of water moving through soils, and through another series of chemical changes known as denitrification.

In denitrification, oxygen is removed from nitrate, reducing it back to the nitrite form and eventually to gaseous nitrogen which is lost to the atmosphere. The bacterial species which are able to carry out this nitrogen cycle are found in all soils, but are most active in soils subject to flooding and water accumulation which bring about anaerobic conditions for the denitrification process.

The use of DWELL with nitrogen fertilizer puts a temporary 'roadblock' in this progression of chemical change. This is done by inhibiting the activity of *Nitrosomonas* bacteria in the conversion of the ammonium to nitrite forms of nitrogen. The result is that the life-span of the ammonium form of nitrogen is prolonged substantially in the soil. At the recommended application rate of 0.5 kg active ingredient per hectare, the activity of *Nitrosomonas* is inhibited without significant effects on the other species of bacteria involved in the soil nitrogen cycle.

Studies by Sommer (1972), in Germany, showed that from the seven nitrification inhibitors tested in culture solutions only etridiazol was toxic to *Nitrosomonas* at 0.5 p.p.m., which was the lowest concentration tested in the experiment. Nitrapyrin was toxic to these bacteria at 1 p.p.m., but not at 0.5 p.p.m. At the highest concentration tested, 25 p.p.m. of etridiazol had no effect on the activity of *Nitrobacter*. This property is desirable because the accumulation of the toxic nitrite ion is prevented.

According to Sommer's soil studies, etridiazol at 1 p.p.m. retarded the nitrification up to 56 days and concentrations of more than 5 p.p.m. resulted in a complete retardation of nitrification up to an incubation time of 84 days. Studies by Rodgers and Ashworth (1982) showed that adding etridiazol at 0.5 mg/kg soil in laboratory completely inhibited the nitrification for 4-5 weeks. In aqueous culture experiments, etridiazol was more bactericidal than nitrapyrin. In the field experiment, injecting etridiazol and nitrapyrin at 1.5 kg/ha with aqueous urea showed that 13 weeks after injection the cross-section treated with etridiazol contained much less nitrate nitrogen than the cross-section without an inhibitor. Little effect of nitrapyrin on nitrification was apparent after 13 weeks.

Laboratory trials in New Zealand (Olin unpublished data, 1974), using etridiazol as a coating on various nitrogenous fertilizers, showed that nitrification can be completely retarded for 4-6 weeks with 1-5 p.p.m. of the

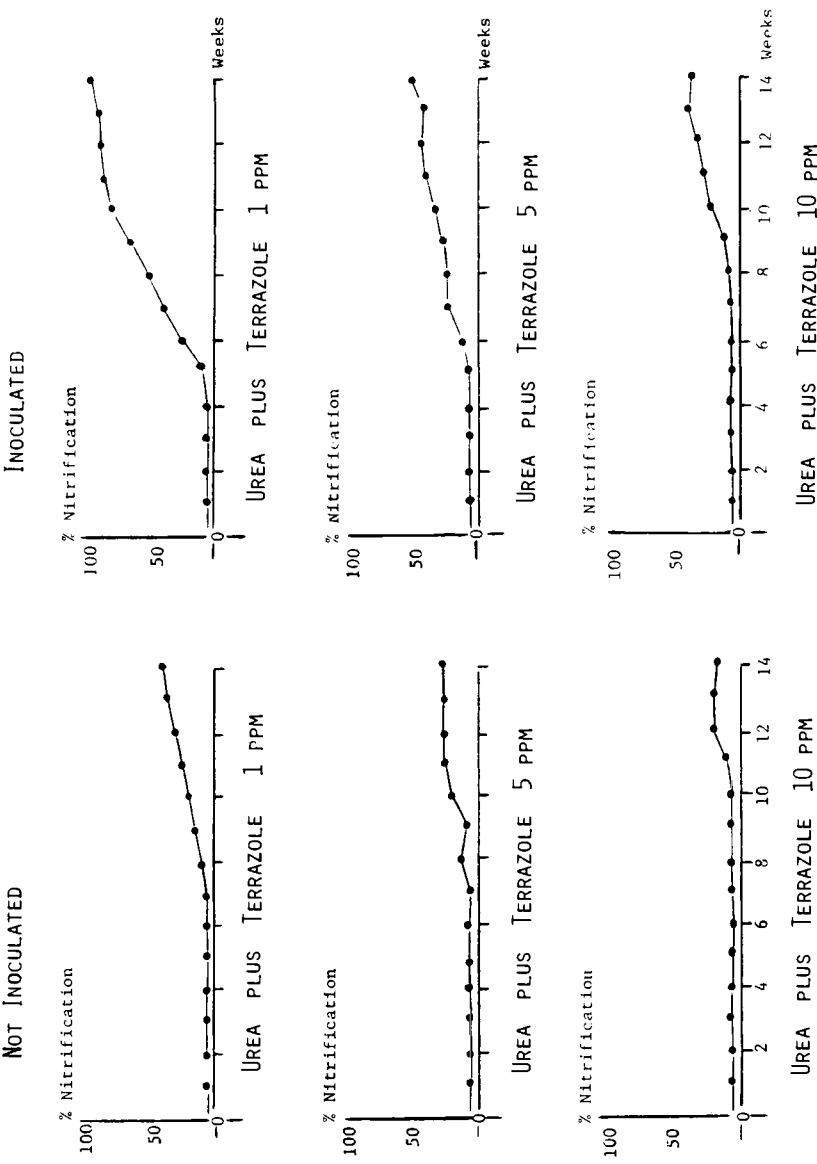


Figure 4.2 Effects of various rates of Terrazole on nitrification of urea equivalent to 200 kg N/ha

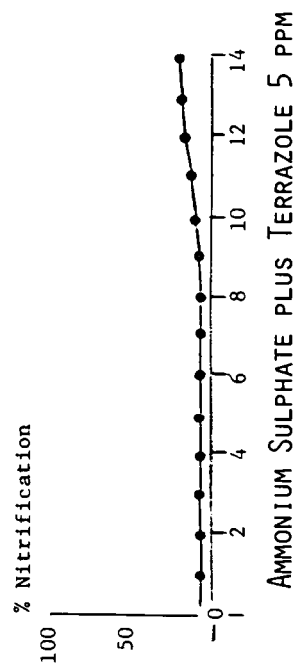
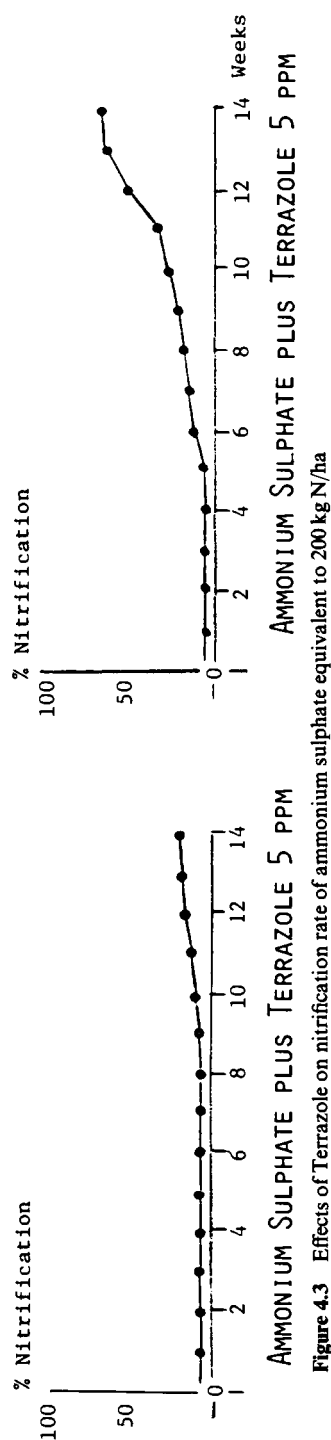
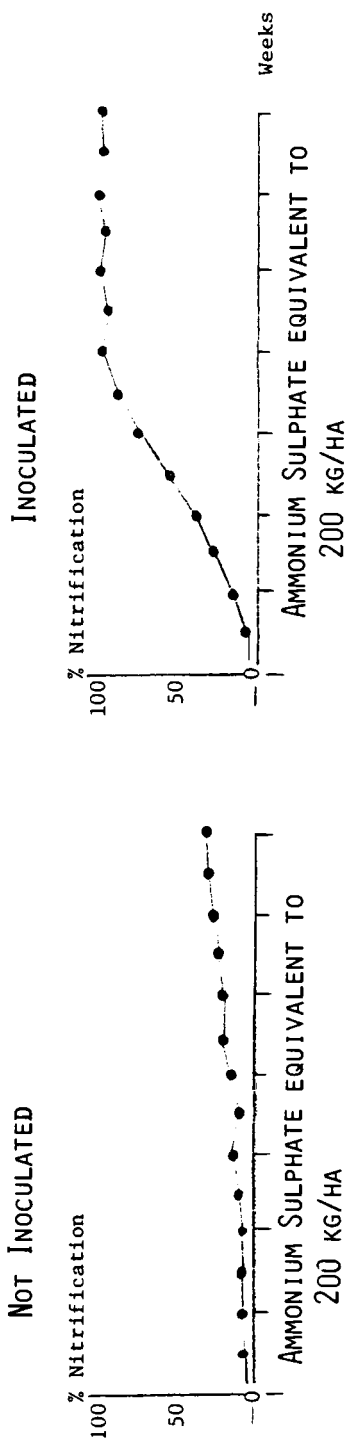


Figure 4.3 Effects of Terrazole on nitrification rate of ammonium sulphate equivalent to 200 kg N/ha

compound (Figures 4.2 and 4.3). Using 10 p.p.m. of etridiazol with urea resulted in a complete retardation of nitrification for 8 weeks, and at 14 weeks nitrification was less than 50%. These trials also compared the nitrification rates of the steamed soil (not inoculated) and steamed soil inoculated with ammonifying and nitrifying organisms. Using 5 p.p.m. of etridiazol with ammonium sulphate at 200 kg N/ha blocked the nitrification completely for 5 weeks. After 12 weeks, nitrification was only 50%.

EFFECTS ON OTHER SOIL MICROORGANISMS

Etridiazol is fungicidal to a number of economically important pathogenic fungi in the soil. Oomycetes, especially *Pythium* spp., are the most sensitive. The lethal concentration for the latter is less than 10 p.p.m. (Halos and Huisman, 1976). Despite this effect, etridiazol is not to be considered a broad spectrum fungicide. Studies by Ercegovich (1976) indicated that the greatest inhibitory action of etridiazole against 13 beneficial soil fungi tested (Table 4.1) was to *Chaetonium globosum* (EC_{50} —28 p.p.m.) and to *Trichoderma viride* (EC_{50} —51 p.p.m.). Its EC_{50} to all other fungi was greater than 100 p.p.m.

Table 4.1 TOXICITY OF ETRIDIAZOL TO FUNGI IN POTATO DEXTROSE AGAR PLATE TESTS

Fungus	EC_{50} (p.p.m.)
<i>Aspergillus flaschentraegeri</i>	~204
<i>A. fumigatus</i>	~100
<i>Chaetonium globosum</i>	~28
<i>Gliomastix convoluta</i>	>125
<i>G. guttiliformis</i>	~125
<i>Humicola grisea</i>	~123
<i>Mortierella alpina</i>	>125
<i>Myrothecium verrucaria</i>	>125
<i>Penicillium chrysogenum</i>	~125
<i>P. notatum</i>	>125
<i>Scopulariopsis brevicaulis</i>	~112
<i>S. koningii</i>	>125
<i>Trichoderma viride</i>	~51

Source: Ercegovich (1976).

In view of this low order of toxicity of etridiazol to these fungi, it is unlikely that this fungicide would upset the dynamics of antagonism attributed to various fungi in soil (*Aspergillus flashchentraegeri*, *Gliomastix guttiliformis*, and *T. viride*); decomposition of cellulose, pectin and xylan (*C. globosum*, *G. convoluta*, *Humicola grisea*, *Myrothecium verrucaria* and *Scopulariopsis koningii*); decomposition of chitin (*Mortierella alpina*); decomposition of lignin (*H. grisea* and *S. koningii*); production of extracellular enzymes (*A. fumigatus*, *G. guttiliformis* and *M. verrucaria*); and the production of antibiotics (*Penicillium chrysogenum* and *P. notatum*). The results of the other soil microorganisms are listed in Table 4.2.

Although etridiazol displayed relatively high toxicity to some of the species

Table 4.2 ESTIMATED LETHAL CONCENTRATION (EC_{50}) OF ETRIDIAZOL TO SOME MICROORGANISMS IN BROTH CULTURE

Organism	EC_{50} (p.p.m.)
ACTINOMYCETES	
<i>Actinomyces streptomycini</i>	10
<i>Nocardia corrallina</i>	> 120
BACTERIA	
<i>Achromobacter metalcaligenes</i>	10
<i>Bacillus cereus</i>	36
<i>B. pasteurii</i>	4
<i>B. subtilis</i>	3
<i>Corynebacterium barkeri</i>	39
<i>Escherichia coli</i>	58
<i>Flavobacterium dehydrogenans</i>	17
<i>Hydrogenomonas rhodochrocos</i>	> 120
<i>H. eutropha</i>	8
<i>Pseudomonas facilis</i>	18
<i>P. fluorescens</i>	141
<i>Rhizobium japonicum</i>	6
<i>Rhizobium</i> spp.	6
YEAST	
<i>Candida albicans</i>	16
<i>Hansenula anomala</i>	10
<i>Rhodotorula</i> spp.	> 60

Source: Ercegovich (1976).

of bacteria, as indicated by the EC_{50} values for *Bacillus subtilis* (3 p.p.m.), *B. pasteurii* (4 p.p.m.), *Rhizobium japonicum* and *Rhizobium* spp. (6 p.p.m.) and *Hydrogenomonas eutropha* (8 p.p.m.), the toxicological activity of etridiazol toward bacteria appears to be one of retardation of growth (bacteriostasis) rather than a high degree of bacteriolysis (destruction of bacteria). After an initial reduction in growth of these bacteria, their numbers remained relatively high and constant through subsequent increases in the concentration of etridiazol in the growth medium. These results are in agreement with those of Rodgers and Ashworth (1982) on *Nitrosomonas* spp., who showed that in the field etridiazol injected at 1.5 kg/ha with aqueous urea did not affect the numbers of these bacteria.

On the basis of the stimulatory effect of low concentrations of etridiazol to *Achromobacter metalcaligenes*, *Arthrobacter globiformis*, *Azotobacter beijerinckii*, *Flavobacterium dehydrogenans*, *Pseudomonas facilis* and *Ps. denitrificans*, it appears that etridiazol may have a unique biochemical effect toward Gram-negative bacteria. Of even more interest is the paradoxical effect observed with *B. pasteurii*. Doses of 1.9 and 3.8 p.p.m. of etridiazol were increasingly more toxic to *B. pasteurii* until the effect began to ameliorate at 7.5 p.p.m. and was completely reversed at 30 p.p.m. Sixty p.p.m. of etridiazol caused a still greater rate of growth of *B. pasteurii*.

Based on these results, Ercegovich (1976) concluded that etridiazol is bacteriostatic rather than bactericidal, and it is unlikely that it would adversely affect this group of microorganisms in soil at the rate and manner in which it is used for agricultural purposes. In this regard, therefore, it should

not cause drastic changes in some of the major bacterial action in soil, e.g. heterotrophic, symbiotic and non-symbiotic nitrogen fixation; the production of various enzymes, including amylase, decarboxylases, dehydrogenases, pectinase, penicillinase, protease and urease; and the decomposition of organic materials in the soil.

The results of the studies on some of the common yeasts and Actinomycetes in soil (*Table 4.2*) showed that these groups of microorganisms were not adversely affected by the application of commercial rates of the chemical.

EFFECTS ON ENZYMATIC REACTIONS IN SOIL

Studies by Ercegovich (1976) have shown that as much as 125 p.p.m. of etridiazol had no adverse effects on the production and activity of invertase, protease, urease, phosphatase and dehydrogenase in Smithdale sandy loam and Hagerstown silt loam soils. Concentrations of 5 and 25 p.p.m. of etridiazol, in fact, caused an elevation of dehydrogenase activity in these soils. Etridiazol was slightly inhibitory to amylase for a short interval after application, but thereafter was responsible for a pronounced increase in amylase activity in Hagerstown silt loam soil for about 16 weeks. Amylase activity in Smithdale sandy loam soil was not enhanced by the presence of etridiazol; on the contrary, 125 p.p.m. caused a slight decrease in activity, but this effect disappeared in about 21 weeks.

Precursory interpretation of the data indicates that etridiazol had more pronounced effects on catalase activity in soil. At 5 p.p.m., etridiazol caused a decrease in catalase activity in silt loam soil, but an increase in catalase activity in sandy loam soil. In both cases these effects disappeared within a few weeks. The highest rate of application, 125 p.p.m., caused a continued decrease in catalase activity in the silt loam soil. Proper interpretation of results for the true effect of etridiazol on catalase activity in soil are marked by methodology factors.

Etridiazol did not have any long-term effects, i.e. for up to 21 weeks, on the stability or activity of any of the seven enzymes tested. Thus, it is doubtful that this chemical would have adverse effects on the fertility of similar types of soil, as it may be related to the enzyme systems.

Mode of action

Studies by Ercegovich (1976) on the effect of etridiazol on several bacteria in the soil suggest that the compound is bacteriostatic rather than bactericidal. Recent studies by Rodgers and Ashworth (1982) in the UK have also shown that, while in laboratory tests with soils amended with nitrapyrin and etridiazol, the inhibitors were more bactericidal, whereas in field experiments they were more bacteriostatic. The exact mechanism of action of etridiazol on *Nitrosomonas* spp. has not been established. However, studies by Lyr, Laussmann and Caspersen (1975), in Germany, on *Mucor mucedo* showed that several ultrastructural alterations were recognizable at 10 p.p.m. of etridiazol

shortly after incubation. These were demonstrated in the forms of vacuolization of the mitochondrial cristae, invagination of the cytoplasmic membrane and thickening of the cell wall. They also showed that the synthesis of triglycerides and sterolesters was inhibited.

Halos and Huisman (1976), studying the mode of action in four *Pythium* species, observed an inhibition of succinate and malate. Etridiazol did not interfere with oxidative phosphorylation, and evidence suggested a block in the electron transport system. The site of the inhibition was between cytochrome b and c. Lyr, Caspersen and Laussmann (1977) found that etridiazol releases and/or activates phospholipase in cell membrane and mitochondria of *M. mucedo*, leading to changes in cell ultrastructure and growth inhibition.

Factors affecting the persistence and bioactivity of etridiazol in the soil

VOLATILIZATION

The chemical is relatively volatile, having a vapour pressure of 2×10^{-2} mm Hg at room temperature. In a study, Redfield, Gay and Sieckhaus (Olin unpublished report, 1981) determined the rate of volatilization from soil surfaces as a function of environmental conditions. Temperature, humidity, soil moisture conditions and air flow were considered in this study. Results showed that more than 50% of the chemical could be volatilized in as little as 4 h at 26.6°C, 90% soil saturation and 0% r.h. Less than 10% loss occurred at 15.6°C, 60% soil saturation and 50% r.h. The order of importance of the variables was temperature > soil saturation > relative humidity > air flow. Soil type did not seem to be important in the evaporation losses, except in case of the high organic (~85%) muck soil, where volatilization losses were greatly reduced. When the chemical was incorporated in the top 10 cm of soil, no losses occurred over a 24-hour period.

ADSORPTION

Studies by D.T. Roberts (Olin unpublished report, 1982) have shown that etridiazol is strongly adsorbed by the soil organic matter. Comparing the adsorption by organic matter and by clay indicated that adsorption to the organic matter was linear, whereas saturation was reached with clay, which indicates fewer adsorption sites on clay than on the organic matter (*Figure 4.4*). In tests with nine soils collected from various sites in the USA, adsorption of etridiazol was found to be significantly related to the organic matter content ($R^2 = 0.90$). Other components of the soil, including clay, did not influence the degree of fit to the model.

The research also studied the relationship between adsorption and efficacy of the chemical as a nitrification inhibitor in eight of the soils. The nitrification inhibition decreased exponentially with increasing soil organic matter content ($R^2 = 0.94$). In coarse-textured soils, a minimum amount of organic matter appears to be necessary to prevent loss of etridiazol by leaching and/or volatilization.

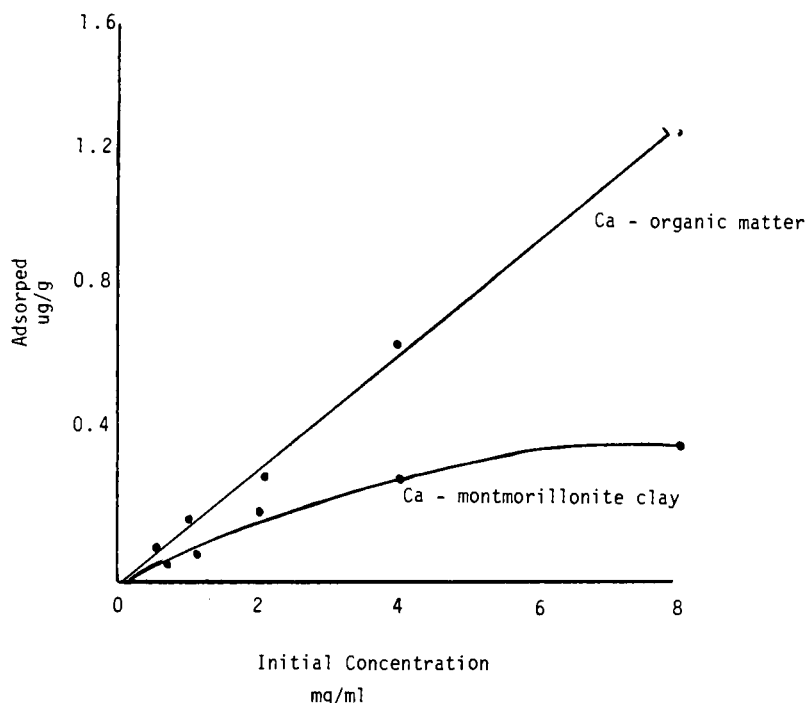


Figure 4.4 Adsorption of etridiazol to calcium-saturated organic matter or montmorillonite clay at pH 6.4

LEACHING

Due to the relatively low water solubility (103–123 p.p.m. at temperatures of 5–37°C) and adsorption to the organic matter, the chemical is not likely to appreciably leach from the soils containing organic adsorptive surfaces. Studies by R.J. Thomas (Olin unpublished report, 1982), using the radio-labelled etridiazol and its acid metabolite, showed that neither the chemical nor its metabolite were found in the leachate of two fine sandy loam soils leached with 0.005 M CaSO_4 . Movement was confined to the first 5 cm section of the column for etridiazol and to the third 5 cm for the metabolite. Movement down the soil column was found to be less in the soils with higher organic matter.

HYDROLYSIS

The effects of temperature, concentration of the chemical and pH were studied in the hydrolysis rate of labelled etridiazol (Wedig, Olin unpublished report, 1982). A hydrolysis half-life of 81–85 days was obtained at the 50 p.p.m. level at 25°C. The acid metabolite (3-carboxy-5-ethoxy-1,2,4-thiadiazole) was the only product of hydrolysis. Increase in hydrolysis was obtained with increase in temperature. At the higher temperature of 45°C, the increase in rate was

affected by the pH of the buffered solutions. The highest rate was obtained at pH 9, with less at pH 3 and lower at pH 6. Besides the acid metabolite, oxalic acid was also identified as a minor product at pH 9, 45°C, and at the 50 p.p.m. level.

PHOTOCHEMICAL DEGRADATION

The photochemical degradation of 3-C¹⁴-labelled etridiazol was determined in the soil (Wedig, Olin unpublished report, 1982). The parent compound was degraded approximately 50% after 48 h of photolysis. A dichlorometabolite (3-dichloromethyl-5-ethoxy-1,2,4-thiadiazole), an acid metabolite (3-carboxy-5-ethoxy-1,2,4-thiadiazole) and carbon dioxide were formed as the result of photolysis. After combustion of the photolysed soil and extraction with methanol and methanol ammonia, 26% of the C¹⁴ material remained after 48 h photolysis.

MICROBIAL BREAKDOWN

Etridiazol is metabolized in soil under aerobic and anaerobic conditions to 3-dichloromethyl-5-ethoxy-1,2,4-thiadiazole (dichlorometabolite) (Wedig, Olin unpublished report, 1982). The acid metabolite (3-carboxy-5-ethoxy-1,2,4-thiadiazole) is also formed under flooded anaerobic conditions. Break-down products such as carbon dioxide and oxalic acid have been identified, which are indicative of cleavage of the 1,2,4-thiadiazole ring. Unidentified soil-bound residues are formed in both aerobic and anaerobic conditions. The aerobic half-life values for both a sandy and a silt loam soil are less than 10 days and the anaerobic are ≤ 5 days at 25°C. Samples of field soil treated with etridiazol were analysed for the parent compound and metabolites. The data from these studies was indicative of the rapid dissipation of the parent compound and its metabolites, i.e. no residues were found at a 9-week sampling.

Samples of field soil from a second study treated with etridiazol were analysed for the parent compound and metabolites. The data from these studies are indicative of the rapid dissipation of the parent and its metabolites. The estimated half-life is less than 1 week at levels of 1 and 10 kg active per hectare.

SOIL TEMPERATURE AND WATER CONTENT

Both of the above factors alter the impact of the chemical and have an interacting effect on the nitrification rate. In one study (Gilmour, 1979), nitrification rates were found to be negligible at 8°C and 15°C irrespective of water content. At 25°C, nitrification rates of 8–16 p.p.m. NO₃⁻-N per week were found at 50% and 75% of water saturation with negligible rates at 25% and 100% saturation. In another study, it was found that, at 19°C, etridiazol was effective over a range of water content of 25–62% of saturation. At the higher temperatures, the efficacy of the chemical was low, especially at the

higher saturation percentages. The chemical was most effective under the cooler and drier soil conditions. As soil temperature and moisture content increase within limits, a reduction in efficacy can be expected.

OTHER FACTORS

Besides the above factors, there are several other potentially important parameters that influence the persistence and bioactivity of etridiazol and other nitrification inhibitors. Some of these factors have been discussed by Keeney (1980) and there are others that require further research. Besides, complex interactions in the soil make the study of one single factor quite difficult. For example, soil pH is a variable that often fluctuates widely when high pH fertilizers are nitrified. Another dynamic factor which is difficult to evaluate is the intrinsic nitrifying ability of a soil.

Factors such as the nitrifier activity, the nitrifier recovery, form of fertilizer and mode of fertilizer application are also important in the bioactivity of the nitrification inhibitors. Each of these factors is, in turn, affected by other variables, bringing about a complex system.

Practical implications

Through nitrification, the relatively immobile NH_4^+ cation is converted to the NO_3^- anion which is freely mobile in the soil solution and is subject to leaching and denitrification. According to Huber *et al.* (1977), of the total nitrogen applied to the soil an average of only 45–50% is taken up by the plant, 25% is changed to organic matter and 25% is denitrified (20%) or leached (5%), *Figure 4.5*. Inhibition or retardation of the nitrification process could diminish the potential for such losses of nitrogen and ultimately increase the efficiency of use of the ammoniacal fertilizers. Etridiazol (DWELL) and other nitrification inhibitors offer a new approach to the nitrogen conservation and management, as discussed below.

(1) *Potential uses of DWELL nitrification inhibitor.* DWELL nitrification inhibitor has the potential for use on any nitrogen-dependent crop which is currently being grown in agriculture. Those crops currently identified and being tested are:

- (a) USA—maize, wheat, sorghum, rice, cotton, barley, oats, turf, potatoes, lettuce and other vegetables;
- (b) Canada—maize, oilseed rape, cereal grains;
- (c) Europe—maize, oilseed rape, cereal grains, sugar beets and vegetables;
- (d) Australasia—maize, cereal grains and vegetables;
- (e) South America—maize, cereal grains;
- (f) South Africa—cereal grains.

In addition, Olin is currently seeking new areas of use for further evaluation. Generally, the greatest potential for benefits from inhibiting nitrification are seen in the following situations (Keeney, 1980; Nelson and Huber, 1980):

- (i) On low organic, coarse soils with pH from 6.2 to 6.8 in areas of moderate to high rainfall;

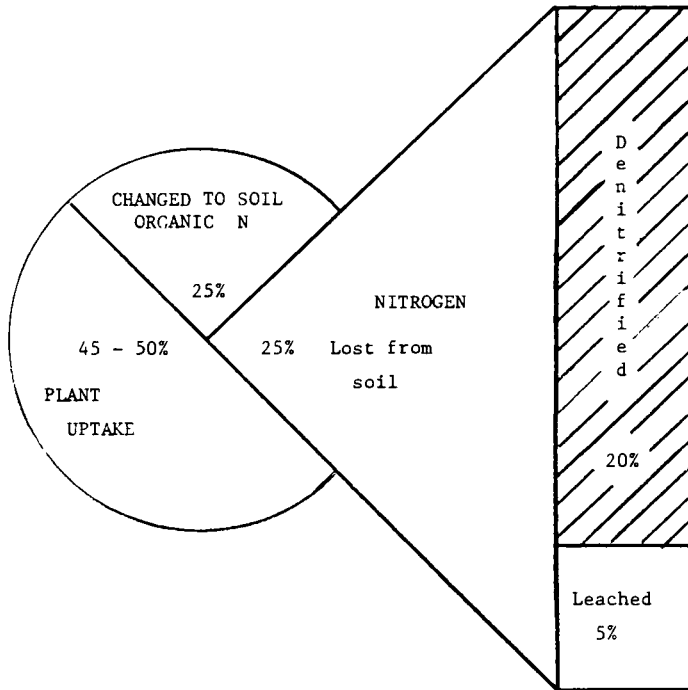


Figure 4.5 Theoretical fate of nitrogen applied to soil (from Huber *et al.*, 1977, by courtesy of Dr D.M. Huber)

- (ii) where there is excessive rainfall or where irrigation is used;
- (iii) where pre-plant applications of nitrogen are made in autumn or early spring;
- (iv) on fine textured soils where water stands after heavy rains;
- (v) on clay pan soils that become saturated due to heavy rains or standing water.

(2) *Versatility of nitrogen applications.* Studies in the Midwestern states in the USA (Nelson and Huber, 1980; Hergert and Wiese, 1980) have shown that nitrification inhibitors can enhance management flexibility in application of nitrogen fertilizers by offering growers the option of autumn or early spring application without suffering severe nitrogen losses. This advantage can best be seen in offering farmers the option of applying nitrogen at a time when nitrogen prices are usually lower and the farmer has more time flexibility.

DWELL offers the user the versatility of using a nitrification inhibitor with this nitrogen programme, regardless of the form of nitrogen being used. When making broadcast applications of fertilizer solutions, DWELL can be mixed in the solution applicator tanks and incorporated (5-10 cm) into the soil during or immediately after application. DWELL may be applied as part of a 'weed and feed' programme, in conjunction with pre-plant incorporated herbicides.

DWELL can also be used in conjunction with dry urea or ammonium sulphate fertilizers. This may be done in one of two ways:

- (a) Dry fertilizers may be broadcast or banded on the soil surface and followed by a broadcast-incorporated spray application of DWELL, alone or in combination with a pre-plant incorporated herbicide.
- (b) Dry urea or ammonium sulphate fertilizers may also be coated with DWELL at a blending facility. Applications on dry fertilizers should be prepared and used on a daily basis and incorporated into the soil immediately.

DWELL is not compatible as a tank mix with anhydrous ammonia. However, it may be applied by using a recently developed injection system which meters the chemical directly into the ammonia line for combined injection. Testing by Olin has shown that the short residence time in the lines does not affect DWELL prior to injection into the soil. This system also offers the option for the farmer to apply selectively a nitrification inhibitor only in those areas of the field which have shown a prior history of severe nitrogen loss.

One new area of nitrogen usage now being evaluated by researchers is liquid manure. It is believed that nitrification inhibitors will become an important factor in the management of this group of nitrogen fertilizers.

(3) *Benefits of nitrogen management using DWELL.* The use of nitrification inhibitors is a rapidly growing area of agricultural practice which can result in improved nitrogen usage, increased compatibility with pest management programmes, decreased labour costs, less damage by certain plant diseases such as stalk rot of maize and the take-all disease of wheat (Huber *et al.*, 1977) and better crop nutrition with less nitrogen loss—all leading to improved plant quality and yields at harvest. These and other benefits have been discussed by Evrard, Partello and Rockwell (1982) and can be summarized as follows:

- (i) conserve nitrogen by reduction of leaching and denitrification;
- (ii) better yields with standard nitrogen rates;
- (iii) uniform nitrogen feeding;
- (iv) flexibility in nitrogen application;
- (v) allows autumn application of nitrogen in spring-seeded crops;
- (vi) reduces nitrite toxicity to plants and animals;
- (vii) saves time, energy and labour through reducing the need for multiple applications of nitrogen;
- (viii) indication of reduced plant diseases;
- (ix) physically compatible as a tank mix with commonly used pre-plant incorporated herbicides;
- (x) effective with ammoniacal forms of nitrogen such as urea, ammonium sulphate, nitrogen solution (28% and 32%) and anhydrous ammonia;
- (xi) reduction of environmental pollution from nitrogen sources.

Field results from Europe

Because of the many factors involved in the activity of nitrification inhibitors the results obtained from different countries have not been consistent. Even within the same country, different locations have produced different results. Because of these variations, several years of testing on several locations in

each country covering the various soil and environmental conditions, as well as fertilizer practices, have been necessary to determine the most optimum conditions for the use of DWELL.

ITALY

Testing started in 1979 in winter wheat and in 1980 in maize. Some of the results have been reported by Calvani (1982) and are presented in *Tables 4.3* and *4.4*. The chemical is now commercially used on maize. All of the nitrogen is applied with DWELL at planting. This practice avoids the costly topdressing applications. Besides this advantage, yield increases of various magnitude have been obtained in some locations, depending on the severity of nitrogen loss from the soil.

Table 4.3 THE EFFECT OF DWELL AND FERTILIZERS ON THE YIELD OF MAIZE IN PIACENZE, ITALY

Fertilizer	Nitrogen (kg/ha)	DWELL 44% (l/ha)	Yield (kg/ha)‡
Ammonium sulphate	220*	0.0	8010 aa
Ammonium sulphate	220*	1.0	9260 cc
Ammonium sulphate + urea	140 + 80†	0.0	9050 cc
Urea	220*	0.0	8240 ab
Urea	220*	1.0	9050 cc
Urea	140 + 80†	0.0	9080 cc
N-solution 30-0-0	220*	0.0	7830 aa
N-solution 30-0-0	220*	1.0	8610 bc
N-solution + urea	140 + 80†	0.0	8950 cc

Source: Calvani (1982), by courtesy of the Eli Lilly Co.

* All of the nitrogen applied at seeding.

† 80 kg/ha nitrogen applied as topdressing.

‡ Means followed by common letters do not differ significantly from one another at $P=0.05$.

Table 4.4 THE EFFECT OF DWELL AND FERTILIZERS ON THE YIELD OF WINTER WHEAT IN PARMA, ITALY

Fertilizer	Nitrogen (kg/ha)	DWELL 44% (l/ha)	Yield (kg/ha)‡
Ammonium sulphate	120*	0.0	4220 aa
Ammonium sulphate	120*	1.0	4840 bc
Ammonium sulphate + urea	80 + 40†	0.0	4910 cc
Urea	120*	0.0	4170 aa
Urea	120*	1.0	4980 cc
Urea	80 + 40†	0.0	4810 bc
N-solution 30-0-0	120*	0.0	4050 aa
N-solution 30-0-0	120*	1.0	4340 ab
N-solution 30-0-0	80 + 40†	0.0	4500 ab

Source: Calvani (1982), by courtesy of the Eli Lilly Co.

* All of the nitrogen applied at seeding in autumn.

† 40 kg/ha nitrogen applied as topdressing in spring.

‡ Means followed by common letters do not differ significantly from one another at $P=0.05$.

AUSTRIA

Testing started in 1980 in winter wheat. As in Italy, the objective was to apply all of the nitrogen with DWELL at seeding. Yield results showed that, besides the practical aspects of this technique, yield increases could be realized under the conditions of the experiments (*Table 4.5*). The product is now commercialized in winter wheat.

Table 4.5 THE EFFECT OF DWELL AND FERTILIZER APPLICATION TECHNIQUES ON YIELD OF WINTER WHEAT IN AUSTRIA

Fertilizer	(kg/ha)*	DWELL (l/ha)	Location I		Location II	
			Yield (kg/ha)	Yield as % of control	Yield (kg/ha)	Yield as % of control
Urea	240 + 0†	1.2	5402	119.9	6178	119.3
Urea	110 + 130†	0.0	5071	113.2	5770	114.4
Mixed fertilizer	180 + 215†	0.0	5254	116.9	5431	104.9
Control	—	—	4914	100.0	5177	100.0

Source: after Dr Karl Lueger, by courtesy of the Kwizda Co.

* Equivalent to 110 kg N/ha.

† Application at seeding + topdressing in spring.

UNITED KINGDOM

Testing started in 1980 in winter wheat and onions. The results with winter wheat are presented in *Table 4.6*. Differences were observed, due to the location and fertilizer application techniques. In general, application of all of the nitrogen with DWELL at seeding was not desirable. It was also clear that using one-half of the normal nitrogen with DWELL did not produce the expected yield. There was some increase in yield with DWELL under the normal practice of fertilizer application, that is, part at seeding and part at topdressing, but the differences were not statistically significant.

Table 4.6 THE EFFECT OF DWELL AND FERTILIZATION TECHNIQUES ON THE YIELD OF WINTER WHEAT FROM THREE SITES IN THE UK

Nitrogen treatment*	DWELL 25% EC		Yield		
	(l/ha)	Site I	Site II	Site III	
No nitrogen	0.0	1.9	2.2	5.1	
All nitrogen at seeding	0.0	3.2	4.0	4.7	
All nitrogen at seeding	2.5	3.6	4.6	4.9	
All nitrogen at seeding	5.0	4.0	4.0	5.0	
Half nitrogen at seeding	0.0	3.6	2.7	5.6	
Half nitrogen at seeding	2.5	3.2	3.4	5.5	
Nitrogen at seeding + topdressing	0.0	4.9	5.8	4.9	
Nitrogen at seeding + topdressing	2.5	5.7	6.0	5.0	
LSD at 5%		1.7	1.6	n.s.	

Source: after D. H. Spencer-Jones and P. Cheale (1981), by courtesy of Midox Ltd

* Total nitrogen per treatment: site I, Suffolk 170; site II, Suffolk 122.5; site III, Kent 86.5 kg/ha.

FRANCE

The most extensive testing has taken place in France. Winter wheat, winter barley and winter oilseed rape, as well as maize, have been used in the tests. Some of the results on winter oilseed rape have been presented (Table 4.7).

Table 4.7 THE EFFECT OF DWELL AND FERTILIZER TECHNIQUE ON THE YIELD OF OILSEED RAPE FROM THREE DIFFERENT LOCATIONS IN FRANCE

Nitrogen (kg/ha)*		DWELL 44% (l/ha)	Yield as percentage of control§		
Autumn	Spring		Location I (Calvieres)	Location II (Cessey)	Location III (Charentilly)
190	+ 0	0	43 bb	80 cc	56 cc
190	+ 0	1	43 bb	76 cc	55 cc
190	+ 0	2	48 bb	76 cc	50 cc
110	+ 80†	0	97 aa	89 ab	83 bb
110	+ 80†	1	96 aa	92 ab	83 bb
110	+ 80†	2	99 aa	100 aa	79 bb
30	+ 160‡	0	100 aa	100 aa	100 aa
30	+ 160‡	1	96 aa	97 aa	96 aa
30	+ 160‡	2	99 aa	103 aa	93 aa
Yield per plot (kg/100 m ²)			1420	2070	2810

Source: after J.L. Leca (1982), by courtesy of Eli Lilly Co.

* As urea.

† One topdressing (normal practice).

‡ Two topdressings.

§ Means followed by common letters do not differ significantly from one another at $P=0.05$.

The results generally indicated that using all of the nitrogen at seeding, even with 21/ha of DWELL, did not carry the crop through the harvest and resulted in low yields. However, using part of the nitrogen at seeding with one topdressing yielded as well as the normal practice of very little nitrogen at seeding and mostly as topdressing, which is the normal practice. It is to be noted that since the seeding takes place in August under relatively warm temperatures, the degradation of DWELL is enhanced and one would not expect an appreciable degree of nitrification inhibition, which would be necessary to reduce the nitrogen loss during the following months after seeding.

Acknowledgements

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PERTURBATIONS IN SOIL ACTIVITY CAUSED BY AGROCHEMICALS

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The widespread utilization of a variety of toxic chemicals in agriculture and industry has led to the contamination of soils with potentially toxic residues. The potential danger of such chemicals and their residues to non-target microorganisms and associated biotic processes has precipitated some unease which has become manifest in increasingly stringent testing programmes for new biocides. Anderson (1978) and Greaves *et al.* (1976) reviewed the wide variety of techniques available for determining the side-effects of pesticides and their residues on soil microorganisms. These techniques fall broadly into two categories:

- (1) Those which estimate the effects of biocides on populations through indirect plating techniques and the more direct procedures of staining and microscopic observation of soil films.
- (2) Those which attempt to determine the effects of biocides on microbial activity and include such procedures as burial of a variety of materials including plant debris, Shirley test cloths and cotton strips; the measurement of soil enzyme activity and soil respiration.

Soil respiration can be assessed either by the measurement of dehydrogenase activity—these enzymes being involved in the oxidative and reductive reactions within the tricarboxylic acid cycle should therefore provide a valid indicator of respiration—or by measurement of gas exchange. The latter has particular advantages when compared to other techniques for measuring microbial activity, as active soil microbial biomass and its composition can be calculated either by the techniques of Jenkinson and Powlson (1976) or Anderson and Domsch (1973; 1974; 1978).

Soil dehydrogenase

Soil dehydrogenase activity can be measured by the conversion of 2,3,5-triphenyltetrazolium chloride (TTC) to 2,3,5-triphenyltetrazolium formazan (TTF) which, in the presence of methanol, generates a red colour whose density can be determined with a spectrophotometer. Soils amended with the herbicides paraquat and 2,4,5-trichlorophenoxyacetic acid (*Figures 5.1 and*

5.2) initially show no reduction in dehydrogenase activity; however, by half-way through the experimental period the level of dehydrogenase activity has markedly decreased, with further significant decreases occurring by the end of the experimental period. A record of the number of microorganisms

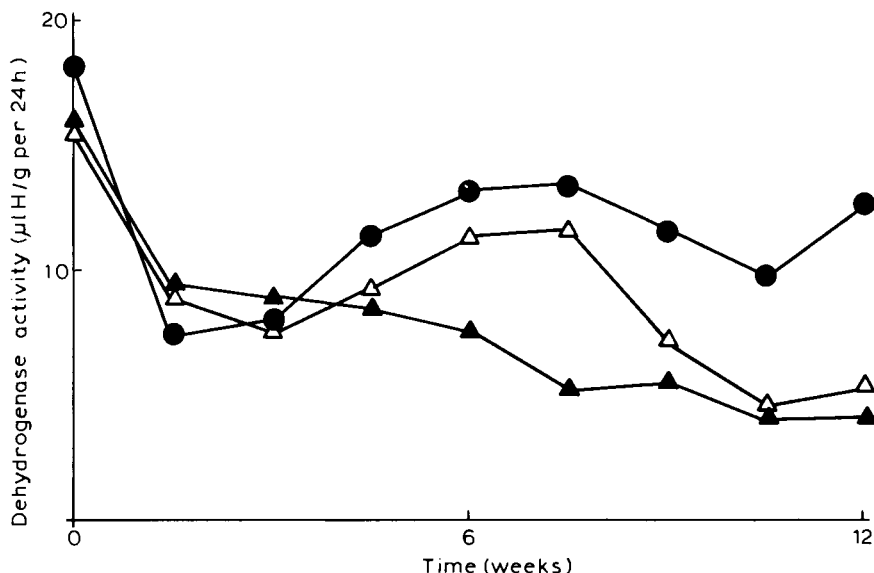


Figure 5.1 The effect of paraquat on dehydrogenase activity: ●, control; △, paraquat at recommended field application; ▲, paraquat at 5 × recommended field application

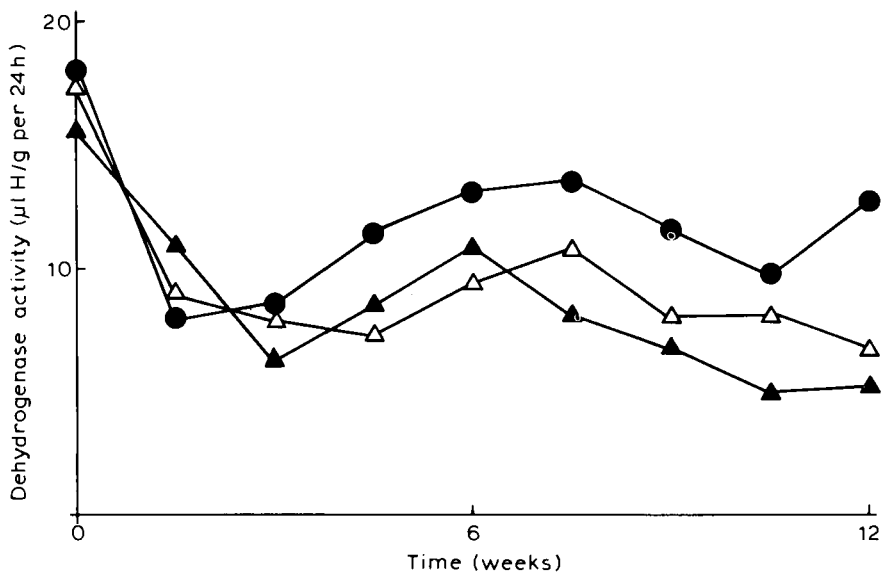


Figure 5.2 The effect of trichlorophenoxyacetic acid on dehydrogenase activity: ●, control; △, trichlorophenoxyacetic acid at recommended field application; ▲, trichlorophenoxyacetic acid at 5 × recommended field application

obtained by indirect plating showed no concomitant decrease in the numbers of soil microorganisms.

Soils which have been contaminated for considerably longer periods with toxic materials will also demonstrate reduced dehydrogenase activity (*Figure 5.3*). Those soils derived from a long-established spoil tip showed little dehydrogenase activity, but soils from the grass-covered areas on the periphery have a significantly greater dehydrogenase activity. The dehydrogenase activity of soil from surrounding ploughed land is also markedly greater than that of soil from the spoil tip. The microbial population of these different soils also varies; samples from the spoil tip demonstrate remarkably little microbial life, just over 100 organisms per gram of soil, while samples from the grass-covered periphery and agricultural land have substantially greater numbers of microorganisms.

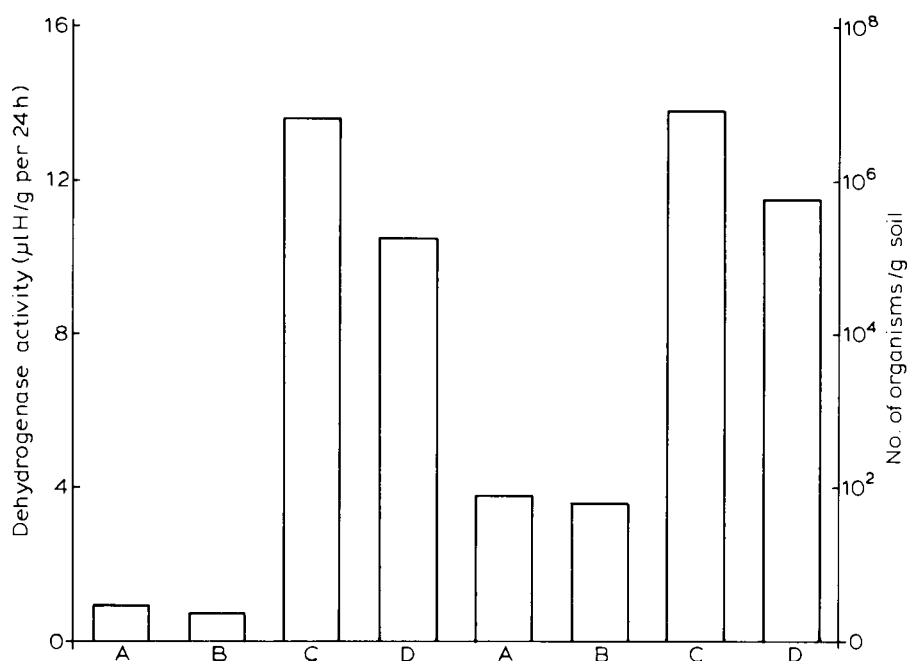


Figure 5.3 Dehydrogenase activity of a spoil tip and surrounding areas. Samples were obtained from: A, surface 5 cm of spoil tip; B, 50 cm below surface of spoil tip; C, surface 5 cm of grass-covered land immediately surrounding spoil tip; D, surface 5 cm of adjoining agricultural land

The results outlined above indicate that dehydrogenase activity is influenced by both microbial numbers and their activity. It is unlikely that the reduction in dehydrogenase activity in soil amended with herbicides is due to microbial mortality, as there was no sudden fall in dehydrogenase activity after herbicide application and little change in the microbial population was observed. Tu and Bollen (1968) also noted that paraquat had little effect on the numbers of soil microorganisms isolated; further, Smith and Lyon (1976) demonstrated that only very high concentrations of paraquat were toxic to soil fungi. Reduction in dehydrogenase activity was therefore probably due

to a reduction in suitable nutrient material, particularly plant root exudates, as both herbicides had destroyed the plant biomass by the end of the experimental period.

However, where soils have been contaminated for considerable periods and microbial numbers are few due to the toxic nature of the chemical residue or a prolonged lack of nutrients, dehydrogenase activity is concomitantly low. Although the dehydrogenase assay gives an indication of microbial activity and is simple to perform, thereby allowing it to be used by relatively unskilled personnel without an array of sophisticated equipment or expensive chemicals, it cannot now be considered a valid indicator of soil microbial activity. Wingfield, Davies and Greaves (1977) have demonstrated some shortcomings in the procedure, but of far greater consequence is the inability of the technique to determine the amount of active microbial biomass or the composition of that biomass without further recourse to enumeration techniques.

Gas exchange

Measurement of oxygen uptake or carbon dioxide evolution from soils has been used to study microbial activity; however, the technique demonstrates considerable inadequacies which have precluded its further use except in specialized circumstances such as the monitoring of residue breakdown through the evolution of $^{14}\text{CO}_2$ from a radioactive-labelled precursor. The specialized techniques of Jenkinson and Powlson (1976) and Anderson and Domsch (1978) have now realized the full potential of utilizing gas exchange, not simply as a technique for assessing microbial activity but as a technique for the quantitative measurement of active soil microbial biomass. Jenkinson and Powlson (1976) demonstrated that the amount of soil microbial biomass could be ascertained by fumigating soils with chloroform and subsequently reinoculating samples to bring about the release of microbial carbon as CO_2 , thereby providing a means of calculating the weight of soil microbial biomass.

Unfortunately the incubation period of 10 days which is required before an accurate estimate of microbial biomass can be obtained precludes use of the technique, as this period is rather too long to readily determine the rapid effects which biocide applications can have on soil microbial biomass. The techniques of Anderson and Domsch (1973; 1978), however, provide a quantitative assessment of soil microbial biomass and its composition within 24 h, thereby providing an accurate determination of the effects of pesticides on the soil microbial biomass within a relatively short time period.

The procedure employed is comparatively simple to perform:

- (1) Determine the optimum glucose concentration above which no significant increase in respiration can be observed.
- (2) Determine soil biomass by measuring CO_2 produced from soil. Substitute readings into the formula $X = 40.04Y + 0.37$ (Anderson and Domsch, 1978), where X = mg microbial carbon per unit soil and Y = ml CO_2 per unit soil.
- (3) Determine the optimum antibiotic concentrations (streptomycin and actidione).
- (4) Determine the percentage of bacteria to fungi by reference to CO_2 production from soils amended with antibiotics.

The incorporation of glucose into soils encourages soil microorganisms to respire at or near their maxima, ensuring that only living, non-resting microorganisms are assessed. Although other carbon substrates have been evaluated, glucose is preferred as it satisfies a number of important provisions. It is water soluble, allowing rapid dispersion through the soil environment and thereby reaching the maximum number of microorganisms in the minimum time period; the molecule is of sufficiently complex structure to prevent its breakdown by free soil enzymes. Further, it is readily utilized by the vast majority of soil microorganisms, is relatively non-toxic, is easily obtained in a pure form and does not affect such important soil parameters as pH. Only CO₂ readings taken within the first few hours may be utilized, as the addition of glucose encourages the expansion of the microbial population which can be seen as an increase in CO₂ evolution (*Figure 5.4*) derived from fresh microbial biomass.

The formula $X = 40.04Y + 0.37$ is derived from a regression line, for a wide variety of soils of mg biomass, as determined by the fumigation technique of Jenkinson and Powlson (1976) against ml CO₂ evolved at 22°C; therefore, all biomass determinations using the Anderson and Domsch (1978) procedures must be carried out at 22°C.

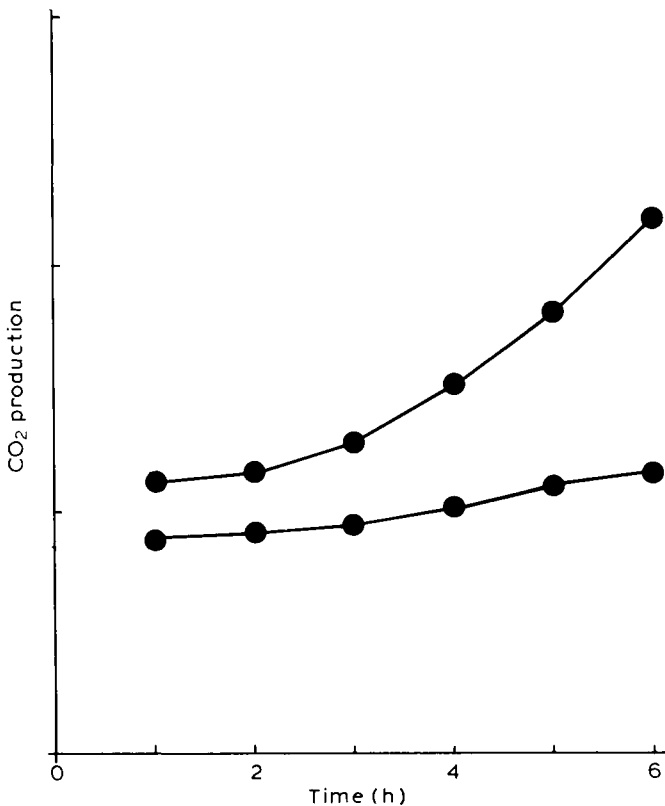


Figure 5.4 The evolution of CO₂ from two different soils amended with glucose

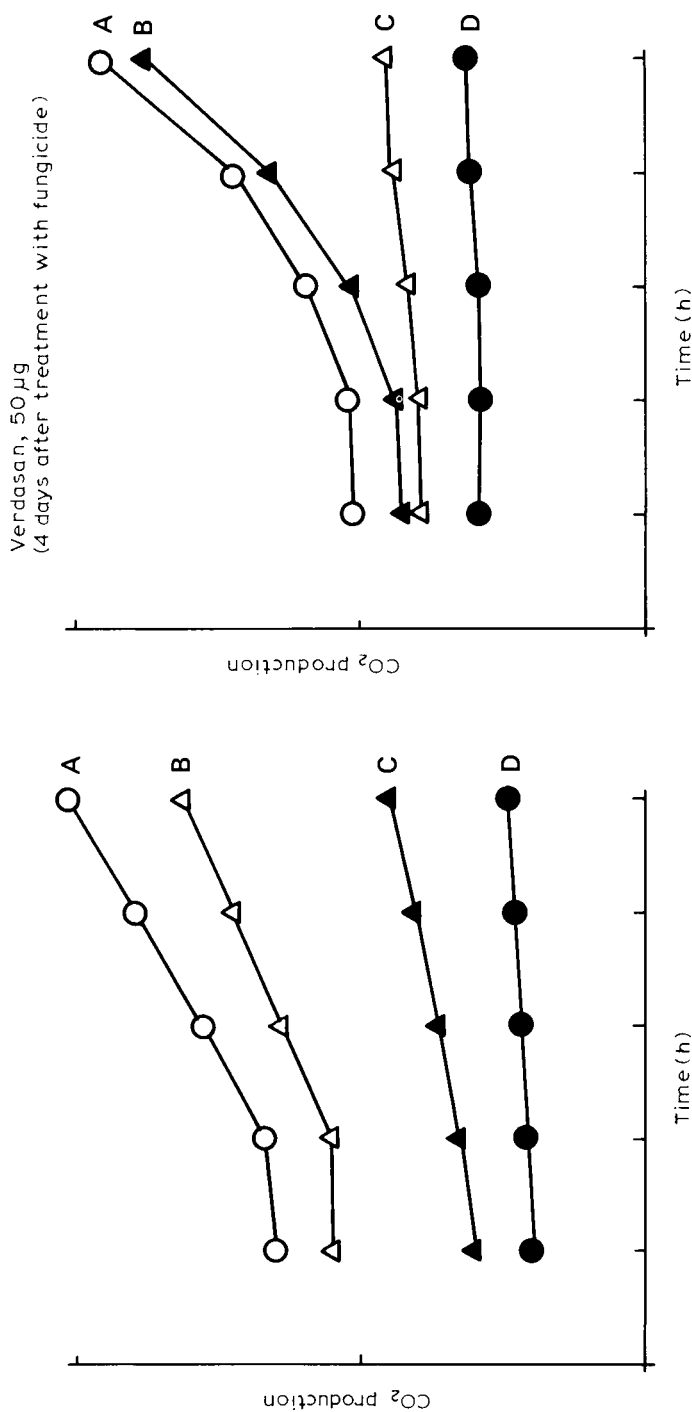


Figure 5.5 The effect of antibiotic inhibitors on CO₂ evolution from soils amended with glucose: A, soil with no inhibitor added; B, soil amended with streptomycin; C, soil amended with both actidione and streptomycin (after Anderson and Domsch, 1974)

$$100 \frac{(A - B)}{(A - D)} = \% \text{ Bacterial contribution}$$

$$100 \frac{(A - C)}{(A - D)} = \% \text{ Fungal contribution}$$

The antibiotics chosen to apportionate the composition of the soil microbial biomass are particularly selective: streptomycin inhibits the synthesis of proteins in prokaryotic cells by interfering with gene transcription, while actidione, in a similar manner, inhibits protein synthesis in eukaryotic cells. The shape of typical curves which can be obtained after the addition of antibiotics is shown in *Figure 5.5*. The relative contributions of bacteria and fungi can be ascertained by the relative reduction in respiration generated by the inhibitor. In uncontaminated soils, the addition of the inhibitor actidione greatly reduces respiration, as the inhibitor diminishes the ability of fungi to respond to the added glucose; however, in soils contaminated with the particularly toxic fungicide verdasan, streptomycin mediates the greatest reduction in respiration, indicating that bacteria are the most important component of the soil microflora after the application of verdasan.

Effect of fungicides on the soil microbial biomass

Soils, some amended with fungicide at concentrations which have been reported by Wainwright and Pugh (1973) to occur after normal fungicide application, were maintained for up to 64 days at original field water potential and 22°C. During this period, the biomass of unamended soils was reduced

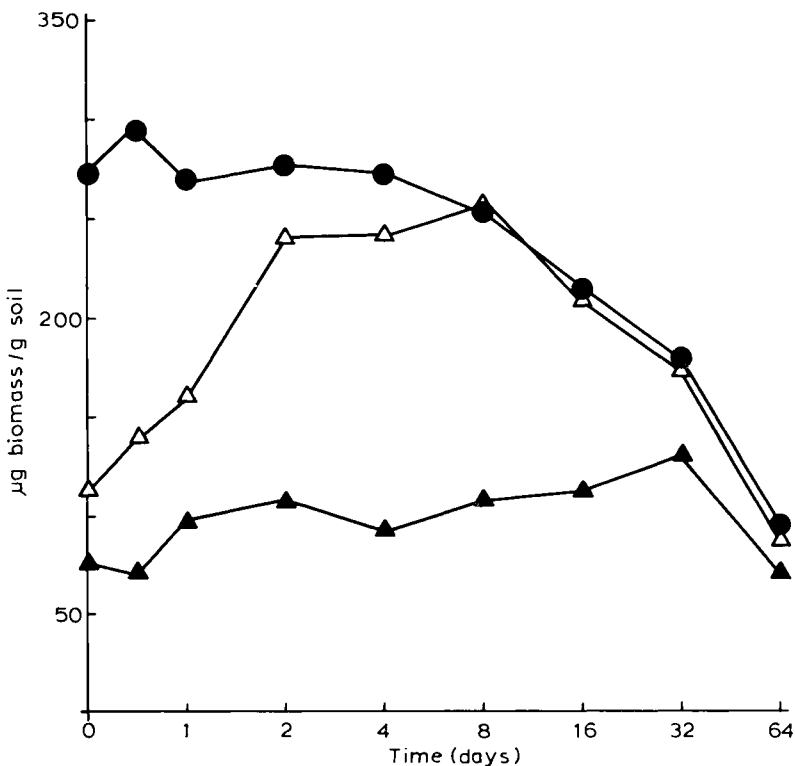


Figure 5.6 The effect of captan on microbial biomass of soil: ●, control; △, 5 µg/g captan; ▲, 50 µg/g captan

by approximately 50%, demonstrating the decline in soil microbial biomass which can occur in soils stored for long periods. All three fungicides severely reduced soil biomass immediately after application.

Figure 5.6 demonstrates that captan reduced the biomass by over half in soil samples amended with 5 $\mu\text{g/g}$ captan (the recommended field application rate is 12 $\mu\text{g/g}$); recovery was complete within 8 days. In samples amended with 50 $\mu\text{g/g}$ captan, recovery was considerably slower, not fully attaining the biomass of control samples by the end of the experimental period. Thiram (Figure 5.7) does not appear to be quite so toxic, as the microbial biomass was

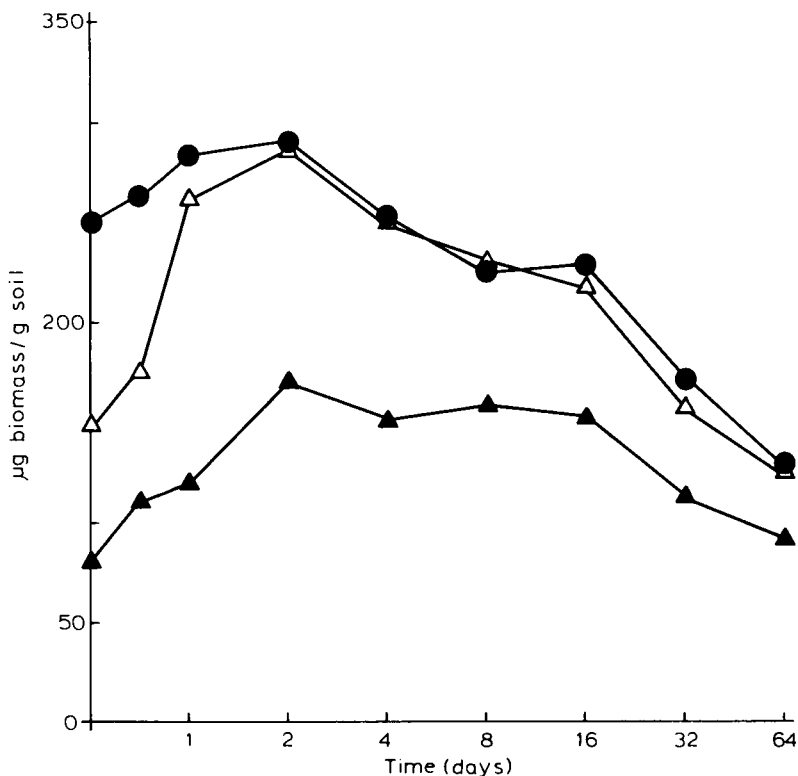


Figure 5.7 The effect of thiram on microbial biomass of soil: ●, control; △, 5 $\mu\text{g/g}$ thiram; ▲, 50 $\mu\text{g/g}$ thiram

not depressed to such an extent by the concentrations of thiram utilized and recovery was also considerably quicker; however, as with captan, the soil microbial biomass did not recover in soil amended with the higher concentration of thiram. Of the three fungicides, verdasan (Figure 5.8) appears to be the most toxic; at a concentration marginally above the recommended field application rate of 4 $\mu\text{g/g}$ soil, the microbial biomass was dramatically reduced and little recovery can be observed throughout the experimental period. As expected, the higher concentrations of verdasan further reduced the soil microbial biomass; however, after only 4 days, the microbial biomass, in soil amended with this particular fungicide, exceeds that present in the unamended

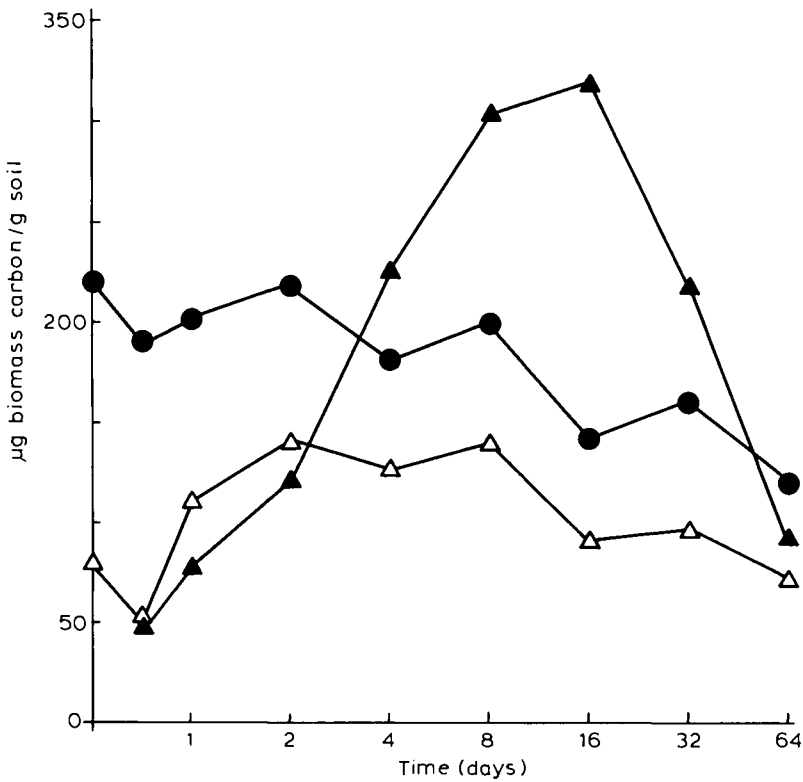


Figure 5.8 The effect of verdasan on microbial biomass of soil: ●, control; △, 5 $\mu\text{g/g}$ verdasan; ▲, 50 $\mu\text{g/g}$ verdasan

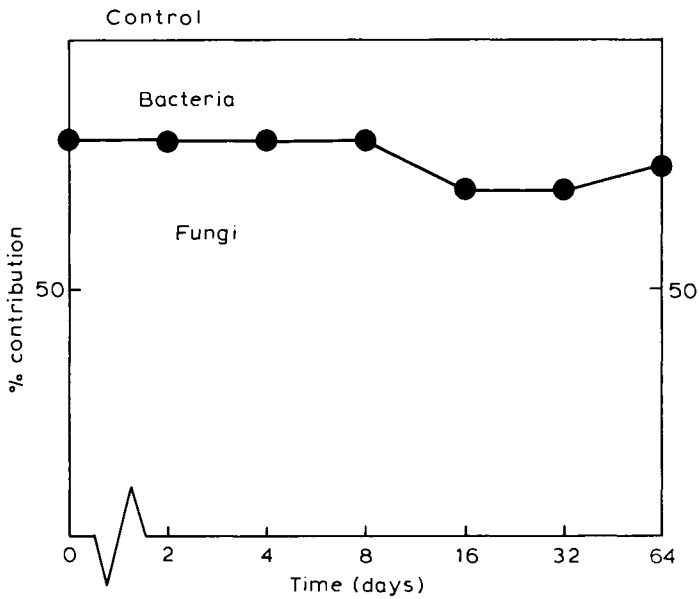


Figure 5.9 The relative contributions of bacterial and fungal populations to soil respiration (continued on pp. 72-74)

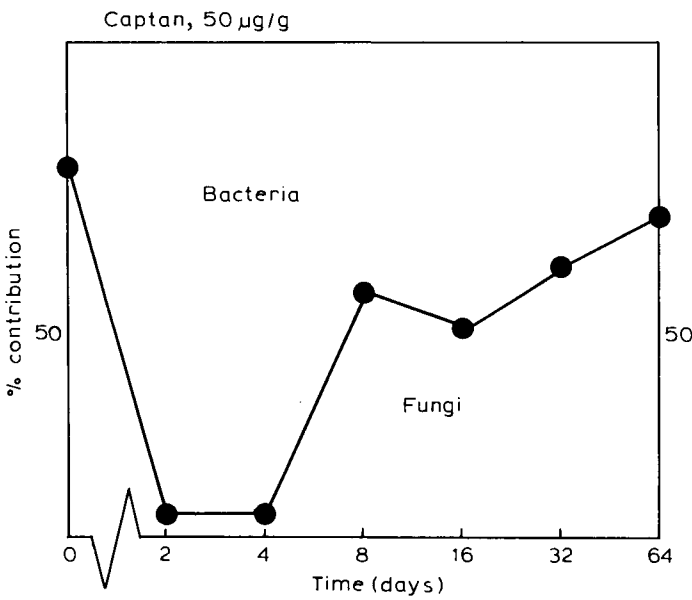
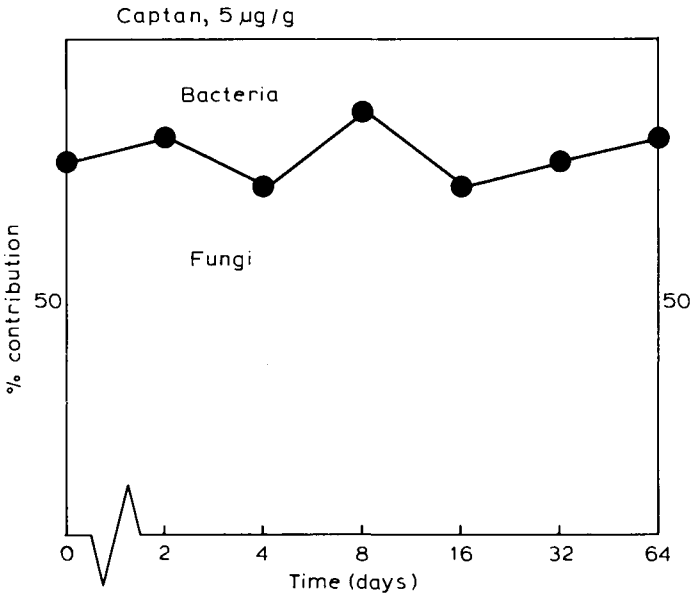


Figure 5.9 continued

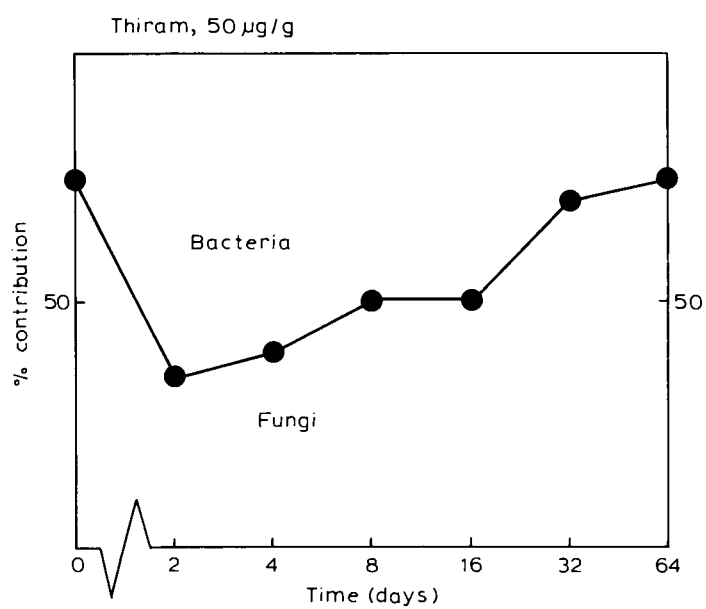
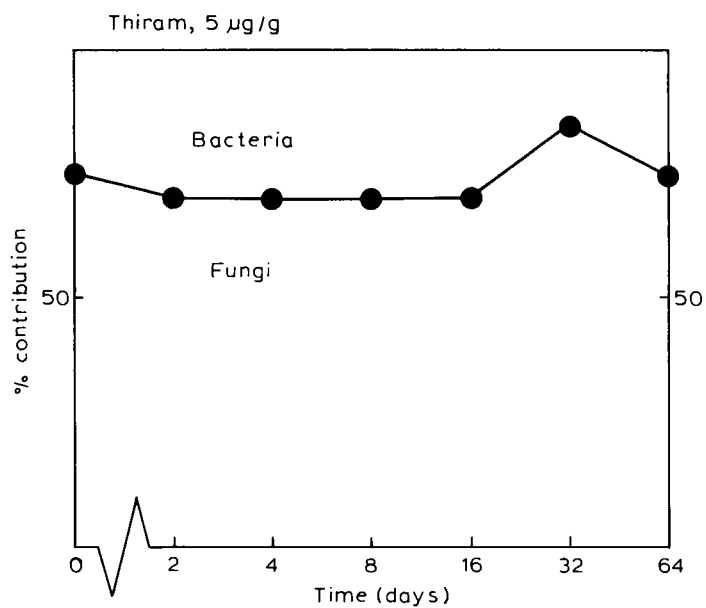


Figure 5.9 continued

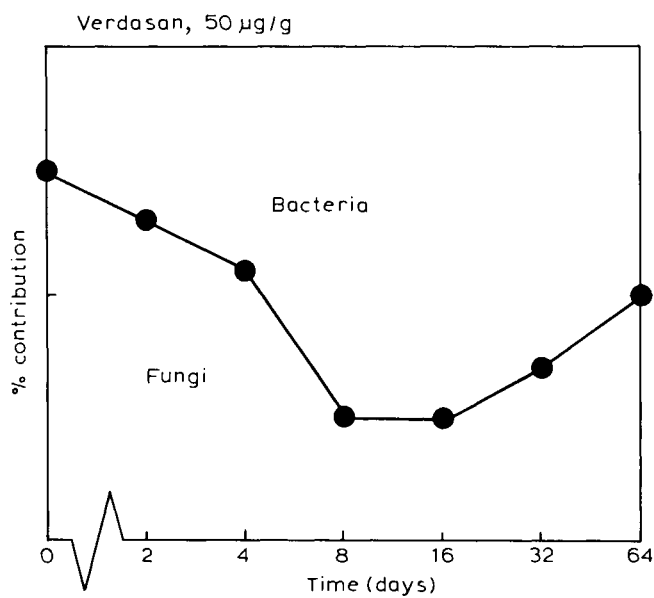
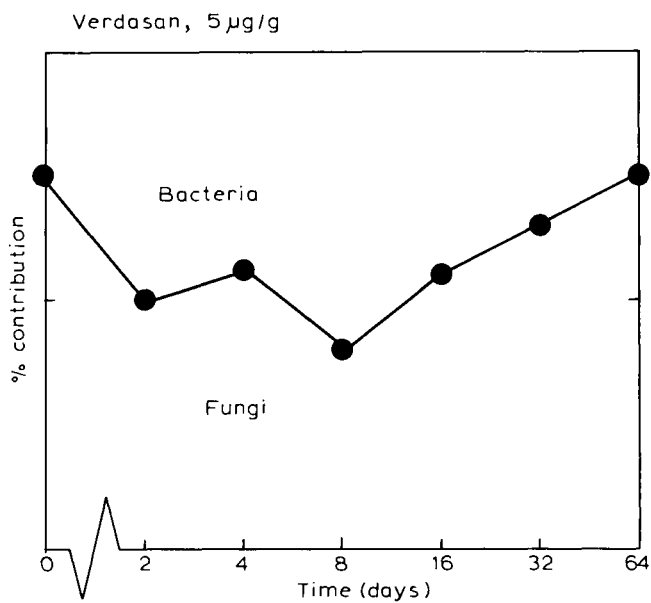


Figure 5.9 continued

soils. The dramatic increase in biomass appears to follow the pattern of a 'flush', with the biomass rapidly climbing to a peak before collapsing.

Although storage of unamended soils may reduce soil microbial biomass, the relative contributions of bacteria and fungi to that biomass remained relatively stable, changing little throughout the experimental period (*Figure 5.9*). The two fungicides captan and thiram can again be grouped together, as neither severely shifted the bacterial to fungal balance of soils amended with concentrations of fungicide which approximate that of the recommended field application rate. The greater concentration of both pesticides does, however, markedly alter the bacterial fungal balance in favour of bacteria, the shift being particularly severe in captan-amended soils. Verdasan, at the lower concentration, altered the bacterial fungal balance in favour of bacteria, but some recovery in that balance to that associated with unamended soils (20/80) can be observed by the end of the experimental period. The higher concentration of verdasan further altered the bacterial fungal balance in favour of bacteria over the same period that the biomass increased, indicating that the increase in microbial biomass was a consequence of an increase in the bacterial population.

METABOLITE LOSS

The unique effects of verdasan on soil microbial populations have also been demonstrated by other investigations (Pugh and Williams, 1971; Kuthubutheen and Pugh, 1979). Anderson, Armstrong and Smith (1981) suggested that this proliferation in the bacterial component of the microbial population was a consequence of nutrient release from moribund fungal mycelium and the utilization of part of the fungicide formulation by bacteria because, unlike captan and thiram, only a very small proportion of verdasan is the active fungitoxic ingredient. However, *Figure 5.10* demonstrates that the organomercury-based fungicide has a particularly dramatic effect on the nutrient status of fungal mycelium compared with other toxic chemicals, as increasing concentrations of verdasan markedly increase the amount of nutrient lost from mycelium. Further, the nutrient loss experienced by fungal mycelium exposed to verdasan is not limited to only one metabolite, but to a variety of metabolites (*Figure 5.11*). Such readily assimilated nutrients normally isolated within the fungal biomass and in limited supply in the soil will become available to other soil microorganisms less sensitive to verdasan. In addition to encouraging efflux of metabolites from fungal mycelium, the fungicide also encourages a particularly rapid loss of metabolites (*Figure 5.12*). The combination, peculiar to verdasan, of the variety of metabolites which may be lost and the rapidity with which such loss can occur probably accounts for the unique effects of verdasan on the soil microflora.

The measurement of metabolite efflux has been employed by a number of workers to ascertain the viability of both plant and fungal propagules. Matthews and Bradnock (1967) noted that seeds demonstrating excessive leakage of metabolites were not only in poor physical condition, but were also liable to colonization by pathogenic organisms. Fungal spores of *Botrytis cinerea* exposed to long periods of leaching lost their viability through leakage and loss of endogenous metabolites required for germination (Blakeman, 1973).

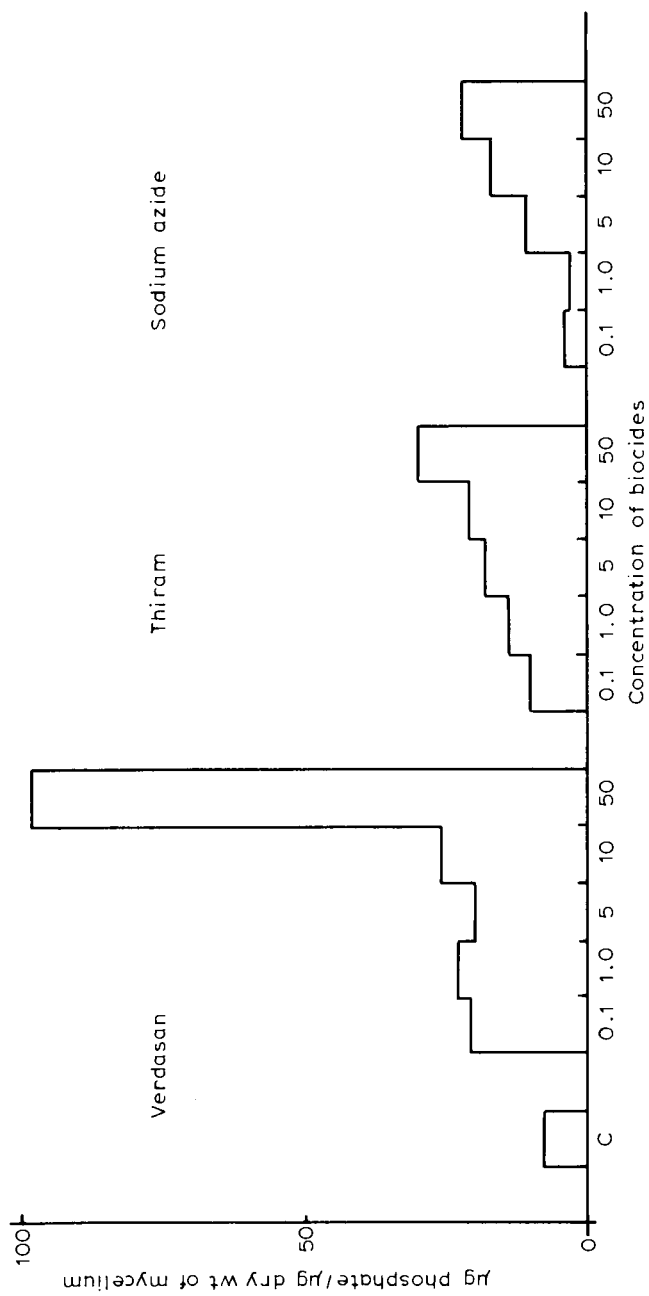


Figure 5.10 The effect of increasing concentration of biocides on the loss of phosphate

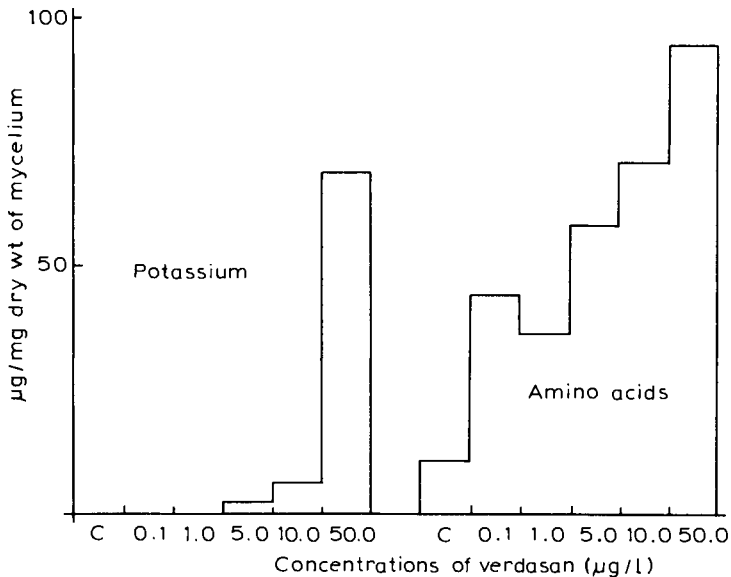


Figure 5.11 The effect of verdasan on the loss of potassium and amino acids

The leakage of metabolites is normally governed by a passive diffusion process, the rate of which can, however, be dramatically increased by a transformation in membrane integrity (Simon, 1974).

Ismail bin Sahid, Lyon and Smith (1981) demonstrated that the process of metabolite leakage could be further enhanced by the action of biocides on both membrane integrity and associated transport processes. Under optimal growth conditions, movement of certain metabolites across the plasma membrane is controlled by an active pump mechanism (Slayman, 1970). The potential of the pump is related to the availability of adenosine triphosphate (ATP); reducing the ATP availability by means of metabolic inhibitors results in a reduction in membrane potential (Slayman, Lu and Shane, 1970). Such a reduction in ATP becomes manifest in the gradual passive efflux of metabolites, as demonstrated by mycelium exposed to the metabolic inhibitor sodium azide (Figure 5.12).

The two fungicides captan and thiram act in a similar manner—captan interferes with the decarboxylation of pyruvate, whereas thiram halts the conversion of acetate to citrate. Both these processes reduce the activity of the tricarboxylic acid cycle with a consequent reduction in ATP formation, thereby accounting for the similarity in the amount and rate of metabolite loss between mycelium exposed to sodium azide and mycelium exposed to thiram. The organomercury fungicide, however, encourages a much more rapid and severe loss of potassium, the magnitude of which is probably the consequence of a breakdown in membrane integrity. This disruption in membrane integrity is probably brought about by the interaction of the organomercury complex with divalent metal ion bridges which link protein subunits together, and so accounts for the unique effects of verdasan on metabolite efflux and, in turn, on the soil microbial biomass.

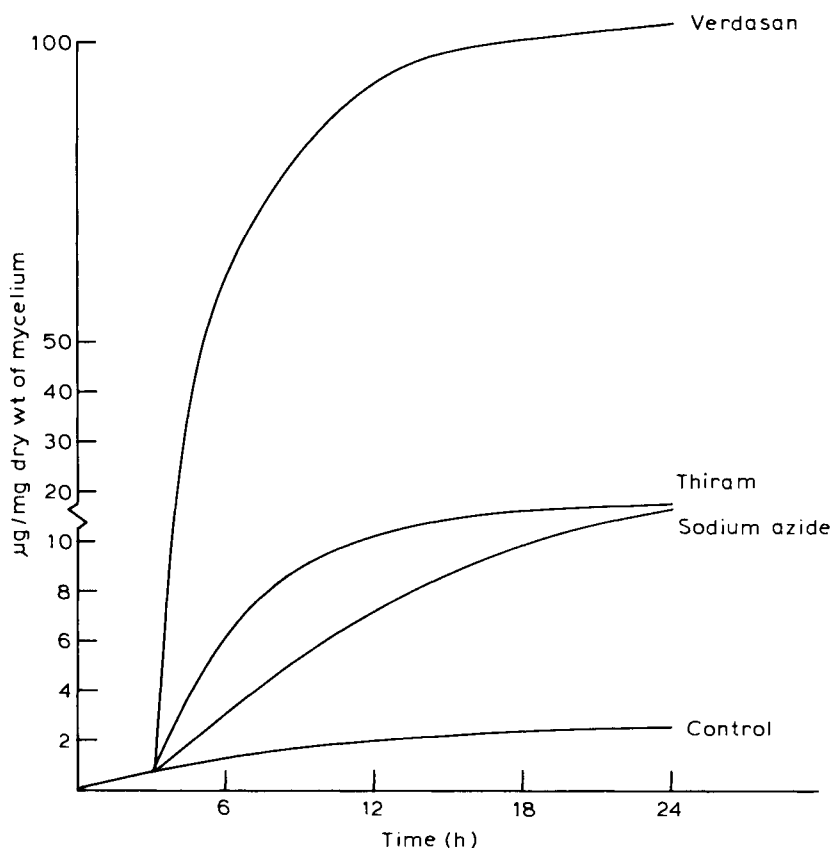


Figure 5.12 The effect of biocides on the rate of phosphate loss

Conclusions

The soil dehydrogenase assay would appear to overcome some of the major pitfalls associated with many other techniques for measuring microbial activity. The assay does not require the addition of specialized substrates which are likely to encourage microbial proliferation, thereby avoiding the use of antimicrobial agents such as toluene which is required in many other soil enzyme determinations. Unlike many techniques which measure the rate of breakdown of particular substrates or baits buried in soils, the dehydrogenase assay will furnish results within a relatively short time period, and the relatively low cost of chemicals and equipment required for the assay allows provision for a large number of samples to be assessed, ensuring adequate replication. However, although the soil dehydrogenase assay can give a useful indication of microbial activity, particularly in soils exposed to extremely toxic materials, the assay provides little indication of the size of the soil microbial population or its composition, as soil samples may have little dehydrogenase activity if few microorganisms are present or conversely soil

samples may demonstrate little activity if the standing population of microorganisms is deprived of suitable nutrient material. This major shortcoming is not restricted to the dehydrogenase assay alone, but is common to all techniques used to measure microbial activity.

Therefore, in studies which assess the effects of biocides on microbial populations, the determination of microbial activity alone, except in more specialized investigations such as the activity of microorganisms involved in nitrification, is of little value as no indication can be gained as to whether particular chemicals are directly toxic to constituent microorganisms or merely alter their circumstances, thereby rendering them inactive but still viable. In soil amended with verdasan, if dehydrogenase activity alone were measured an increase would probably have been observed, yet this biocide has been shown to be among the most toxic and deleterious to certain components of the soil microflora, its use resulting in major changes within the soil environment. The measurement of microbial activity must therefore be supported with further enumeration techniques.

Measuring the maximum activity of soil microorganisms does appear to be of greater value, as any reduction in activity indicates a reduction in the microbial biomass, the extent of which can be easily quantified. The technique is sufficiently sensitive to follow the major changes in soil microbial biomass which can occur after soil amendment with pesticides. In soil exposed to fungicides, these changes can be considerable, supporting the view that contact fungicides appear to be potentially the most deleterious pesticides both to soil microorganisms and associated biotic processes. The assessment of maximum activity after soil amendment with selective antibiotics allows the behaviour of the prokaryotic and eukaryotic components of the soil biomass to be assessed. Such studies are also of considerable importance, as a number of soil biotic processes are commonly associated with one or other of these groups of organisms and soils showing considerable prolonged imbalance in the composition of the microflora may demonstrate impaired fertility. Therefore, although the assessment of soil microbial activity alone may be of little value, those techniques which measure maximum respiratory activity demonstrate considerable potential for estimating the response of the soil microbial population to agrochemicals and so are worthy of continued refinement and investigation.

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FUNGICIDES, FUNGAL POTENTIAL AND SOIL MICROBIOLOGY

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Fungicides are widely used in agriculture around the world, and with ever-increasing demands for more and better food for an expanding world population, the application of fungicides and other agrochemicals will continue into the foreseeable future. They were estimated to be used in the production of half the world's crops more than 10 years ago (Ordish and Mitchell, 1967). The scale of usage can be gauged from the known application rates; thus, in 1971 in the USA alone, more than 50 000 kg of organic mercury were used in agriculture, while about 300 000 kg of organic mercury were used as fungicides in paints (Stoker and Seager, 1976).

Fungicides are designed to kill fungi, and the criterion that 'a desirable soil fungicide is one which selectively kills or inhibits pathogenic fungi in soil when used at concentrations non-toxic to other soil microorganisms' (Corden and Young, 1965) is an unattainable ideal because the cellular processes which are inhibited by fungicides are common to most microorganisms. In this context it must be realized, and it cannot be stated too often, that plant pathogenic fungal species represent only a small proportion of the total number of fungal species. Estimates vary as to the total number of fungi: perhaps as few as 100 000 (Ordish and Mitchell, 1967); perhaps as many as 250 000 (Ainsworth, 1971). Of these, about 100 species are common and serious plant pathogens. Thus, considerably more than 99% of known fungal species are not harmful to plants, and through their decomposition activities are beneficial in nature. Yet they are as susceptible to fungicides as are the plant pathogenic species.

The application of fungicides to a crop is never perfect: some of the material will get into the soil by drifting sprays, or by run-off, or by spillage; some of the more persistent fungicides will remain on leaves until after leaf fall and their incorporation into the litter layer. When the fungicide reaches the soil, what is its likely effect on the soil microbial population and its activities? How does it affect the various soil biochemical processes which the microorganisms activate? Questions such as these must be asked, particularly when there is an extension of the use of fungicides and other agrochemicals on a wider scale throughout the world, as developing countries look for greater food production. The benefits to be derived from the fungicides are readily understood; the question of deleterious effects has to be faced.

Methodology and its rationale

In order to estimate the effects of fungicides on soil populations, various methods can be used which measure different facets of those populations. We have to be most careful in realizing just what we are measuring, and equally importantly what we cannot measure by any given technique. Indeed, the limitations imposed by a technique are not infrequently overlooked; only by developing and expanding a new technique or by using a known technique in new circumstances can its limitations be found. Thus, for bacteria and other unicellular microorganisms, the use of the old and well-known dilution plate technique can give a count of the viable cells which can grow on the medium used and in the environment in which the cultures are incubated. But every agar medium is limiting and selective to a greater or lesser degree. Just as some media allow a broader spectrum of organisms to grow than others, so also the temperature of incubation can encourage some organisms while discouraging others. Yet, as a generalization, most cultures are incubated at 25°C unless thermophiles or psychrophiles are being searched for. But how often is the soil at that temperature? It is a convenient temperature for rapid growth of most common soil microorganisms, even though a different view of those organisms may be obtained if a more natural soil temperature was used.

While dilution plate counts of bacteria will tell us something, within these limitations, about the numbers of viable cells in the soil sample, they cannot tell us anything about the state of activity of those cells at the time of sampling. However, successive sampling over a period of time which covers some change to the environment, such as the application of an agrochemical, can reveal a decrease in numbers, indicating death of cells, or an increase which may be a measure of their activity, with increased metabolism leading to cell division, or it could measure some adverse effect which led to cell division only.

Difficulties such as these in interpreting the results for unicellular organisms become very much greater when filamentous fungi are considered. To count fungal numbers *per se* is meaningless: what are we counting—individual colonies, longer or shorter lengths of mycelium, resting propagules, dispersal spores, or some unknown mixture of several of these? Even the expression ‘individual colonies’ begs the question as to what this refers to: any non-sporing filamentous fungus growing from a single source will in the course of time spread in a three-dimensional way. The colony will eventually die at the centre, so that the margins are then separate and discrete, but all are organically related to each other, i.e. they are clones. When they were still joined together they were one organism, and it can justifiably be argued that through the activity of that organism it has grown to form several new organisms. But at our moment of sampling we do not know if we have several parts of the original colony which we have broken up, or several individuals which have separated through their own activity.

When we consider a filamentous fungus which has sporulated, the meaning of a fungal count becomes even more obscure: has the colony developed from an active piece of hypha, or from some moribund mycelium, or from a spore, of which thousands may have been produced? It has been argued that such

an increase in numbers brought about by sporulation can indicate activity on the part of the fungus (Montegut, 1960), but equally it can be said that sporulation may occur when the fungus is less active, when its nutrient supply is diminishing or changing in character.

For these reasons, counts of fungi are at best only relative, showing a change from one soil or soil-horizon to another, or after a particular treatment compared with a control or pre-treatment situation, and have no absolute values. In some circumstances any changes in counts may indicate a change in activity of the fungi, but these can only be speculative unless other criteria are used simultaneously. To avoid the use of numbers *per se*, or the unfounded implications of activity, the term 'soil fungal potential' was suggested (Pugh, 1963), where the potential was defined as 'active mycelium plus propagules which are the result of previous activity, held dormant by such factors as mycostasis'. The potential can be regarded as a term of assessment devoid of overtones of absolute numbers or of current microbial activity. Changes in the potential, then, are relative, and as such can be sought following a specific treatment, whether soil dilution plates, or soil crumb plates, are used. Obviously with soil dilutions, colonies are counted, while each soil crumb plate is treated as a micro-quadrant, with species recorded as being present or absent, and assessments made on the total number of plates used. In this way, a record can be kept of numbers of different species present, as well as the potential of each species.

Other methods can be used to assess changes in processes occurring in the soil: respiration rates can be measured, but selective inhibition of bacteria, fungi or soil fauna has to be exercised to arrive at a figure for any one group of organisms (Anderson and Domsch, 1974); biomass can be studied (Anderson and Domsch, 1978); lengths of living mycelium can be measured (Nagel-de-Boois, 1971), but they need to be distinguished from dead or inactive mycelium; the use of ^{14}C -labelled substrates can help elucidate the patterns of breakdown of these substrates, and can give an indication of fungal activity (Grossbard, 1969; Jenkinson, 1977). We have examined the amounts of ammonification and nitrification, following the application of fungicides (Wainwright and Pugh, 1973), and the quantification of dehydrogenase to give an indication of soil microbial activity (Smith and Pugh, 1979). The use of such a technique following selective inhibition of part of the soil biota could give a good estimation of activity of the uninhibited section.

Subsequent laboratory-based studies were carried out on pure cultures of fungi. These included:

- (1) The growth rates of three non-mycorrhizal basidiomycetes, *Coprinus comatus*, *Cyathus stercoreus* and an unidentified species 235m, two mycorrhizal species, *Boletus variegatus* and *Paxillus involutus*, and *Phallus impudicus*, which is a species of uncertain status. The fungi were grown in the presence of a range of concentrations of the herbicides mazide (Synchemicals) and paraquat (Gramoxole, ICI), and of the fungicide verdasan (ICI).
- (2) The growth of *Fusarium culmorum* and of two strains each of *F. nivale*, *F. oxysporum* and *F. solani*. Each isolate was grown in the presence of different concentrations of the herbicides 2,4,5T and paraquat and the fungicides captan (ICI) and verdasan (ICI).

Results

Following a single application of the fungicides used, two very different trends were found. In *Figure 6.1* it can be seen that the fungal potential, whether assessed as numbers of colonies or of species, showed an immediate steep decline, followed by a slow rise. The pre-treatment level had not been regained in 6 weeks. Dilution plate counts of the bacteria increased to a peak in 14 days and then declined until, after 6 weeks, the numbers were approaching the pre-treatment level. Within these trends, the four fungicides used showed different degrees of effect, within the sequence dicloran < captan < thiram < verdasan. These fungicides were applied at monthly intervals for a year, and *Figure 6.2* shows that thiram, and especially verdasan, greatly reduced the summer peak in fungal potential.

The short-term effects of the fungicides on the bacteria produced marked changes in ammonification and nitrification. Wainwright and Pugh (1973) showed that increasing concentrations of the fungicides raised the levels of ammonification, and decreased the levels of nitrification in the soil over a 4-week period. Again, the effectiveness of the fungicides varied: verdasan at 10 µg/g produced an effect which was similar to thiram (ICI) at 100 µg/g and captan at 250 µg/g of soil. The general effects are shown in *Figure 6.3*.

The long-term effects of the fungicides was most evident in the species-composition of the fungal flora. This effect was greatly enhanced following successive treatments. Two distinct groups of fungi were found:

- (1) Those which disappeared from treated soils, which were obviously intolerant of the fungicides being tested.
- (2) Those which were present after treatment. These may have been tolerant, and thus able to survive the treatment and subsequently to be able to colonize the treated soil from the surviving inoculum; or they may have been intolerant and only able to recolonize the soil once the toxic effects of the treatment disappeared.

By growing these fungi in a range of concentrations of the fungicides, we have been able to separate the tolerant from the intolerant but rapidly recolonizing species.

Some of these fungi are listed in *Table 6.1* following treatment with thiram and with verdasan. One feature of especial interest concerns the different reactions of two species of *Trichoderma*. While *T. hamatum* was tolerant to both fungicides, *T. viride sensu stricta* was tolerant to verdasan. This emphasizes the need to identify these species and not to lump them, as so often has happened, as *T. viride sensu lata*.

From the fungi which were tolerant to verdasan, *Chrysosporium pannorum* was selected for further study. In its temperature relationships, it germinated and grew satisfactorily at 5°C, and showed optimum growth at 18°C. In the presence of verdasan, the growth of *C. pannorum* on agar media was little affected up to 200 p.p.m. of the fungicide, but there was no growth at 800 p.p.m. In liquid media, the reduction in growth occurred at much lower concentrations, with little increase in dry weight at 100 p.p.m. A 50% reduction in growth occurred at a concentration of about 3 p.p.m.

From an analysis of the toxicity remaining in the liquid growth medium, we were able to show that the fungus was able to detoxify the fungicide within

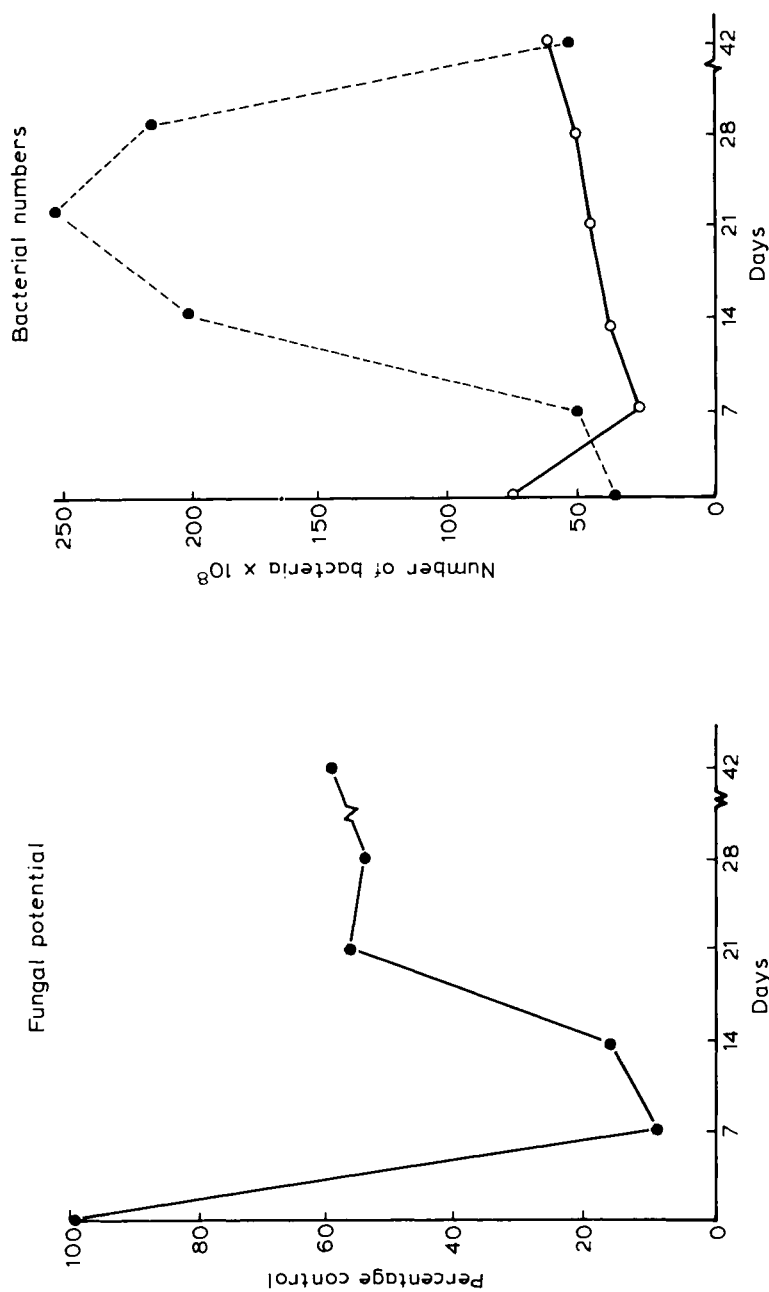


Figure 6.1 Response of fungal potential and bacterial numbers to one application of verdasan at 250 $\mu\text{g/g}$ soil (after Williams, 1973): ●—●, treated soil; ○—○, control soil

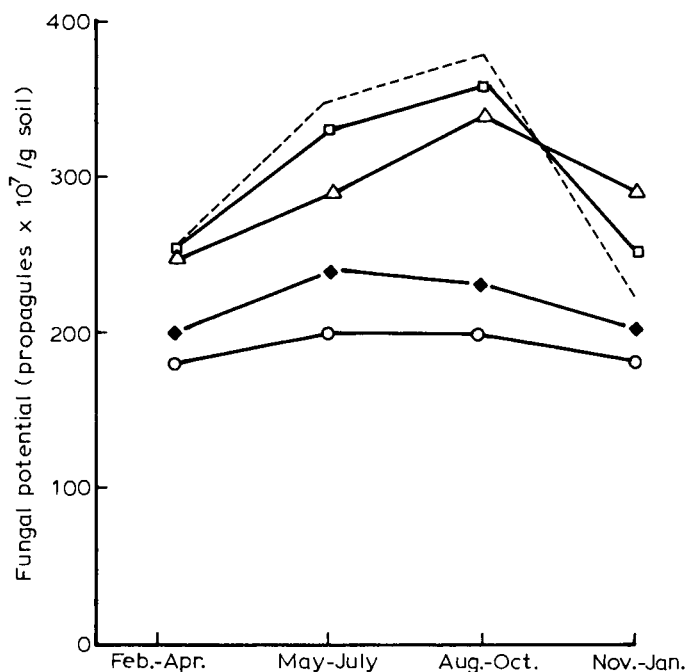


Figure 6.2 Reduction in fungal potential following regular applications of four fungicides (from Kuthubutheen, 1977): ---, control; □—□, dicloran; △—△, captan; ◆—◆, thiram; ○—○, verdasan

6 days, when the initial concentration was 12.5 p.p.m., and in 13 days, when it had been 100 p.p.m. This detoxification was correlated with a loss of mercury from the liquid, and this mercury was subsequently found within the mycelium of the fungus (Williams and Pugh, 1975).

The growth of the Basidiomycetes showed that the mycorrhizal species were less tolerant of the agrochemicals used than were the non-mycorrhizal species (Pugh and MacDonald, 1980). With mazide, the non-mycorrhizal species were all growing at 8000 p.p.m., while growth of the mycorrhizal species had ceased; with paraquat, basidiomycete 235M and *Coprinus comatus* grew at 100 p.p.m., while the other four species did not exceed 5 p.p.m.; in the presence of verdasan, the mycorrhizal species did not grow, *Phallus* showed slight growth at 0.25 p.p.m., and three non-mycorrhizal species grew at 0.75 p.p.m. (*Cyathus*), and above 1.0 p.p.m. (235M and *Coprinus*).

The preliminary results obtained with species of *Fusarium* showed that *F. nivale* ceased growth in the presence of 1000 mg/l of paraquat, and of 700 mg/l of 2,4,5-T. The other three species, *F. culmorum*, *F. oxysporum* and *F. solani* all grew at these concentrations. All four species showed growth at 10 mg/l of verdasan and at 100 mg/l of captan. With both herbicides and fungicides some differences were obtained in the levels of tolerance exhibited by strains of *F. solani*, while the strains of *F. nivale* differed in the presence of 2,4,5-T.

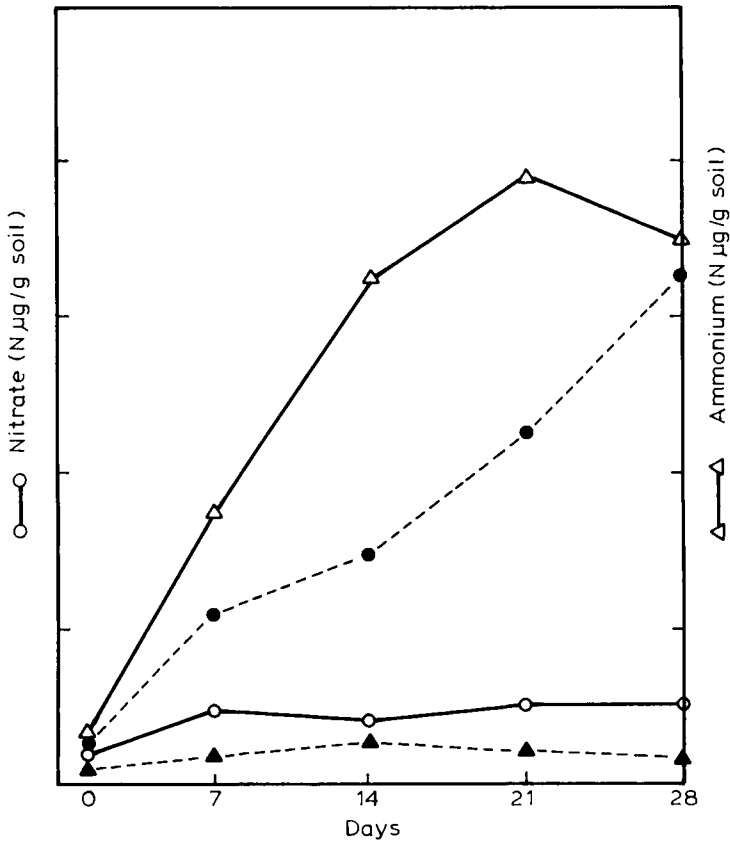


Figure 6.3 Generalized effect of fungicide treatment on nitrification (○—○) and ammonification (△—△) obtained with concentrations of verdasan at 10 µg/g soil, thiram at 100 µg/g soil and captan at 250 µg/g soil—controls in black symbols (after Wainwright and Pugh, 1973)

Table 6.1 TOLERANT AND INTOLERANT BUT RAPIDLY RECOLONIZING FUNGI PRESENT IN SOIL AFTER TREATMENT WITH FUNGICIDES

Fungicide	Tolerant species	Recolonizing species (both treatments)
Thiram	<i>Apiosordaria verruculosa</i> <i>Botryotrichum piluliferum</i> <i>Cladosporium cladosporioides</i> <i>Mortierella minutissima</i> <i>Penicillium chrysogenum</i> <i>Trichocladium asperum</i> <i>Trichoderma hamatum</i> <i>Zygorrhynchus moelleri</i>	<i>Botryotrichum piluliferum</i> <i>Gliocladium roseum</i> <i>Gliomastix murorum</i> var. <i>felina</i>
Verdasan	<i>Chrysosporium pannorum</i> <i>Cladosporium cladosporioides</i> <i>Mortierella minutissima</i> <i>Trichocladium asperum</i> <i>Trichoderma hamatum</i> <i>Zygorrhynchus moelleri</i>	<i>Humicola fusco-atra</i> <i>Sepedonium chrysospermum</i> <i>Trichoderma viride</i>

Source: Kuthubutheen and Pugh (1979).

Discussion

The application of fungicides to the soil has a number of effects which concern both bacteria and their activities as well as the fungi. When susceptible fungi are killed, their remains form substrates which can be utilized by tolerant fungi and bacteria. The immediate result which is apparent is the flush of bacteria during the 7–14 days following the fungicide application. Within this increase in bacterial numbers there must be a differential effect, because the bacteria concerned with the cycling of nitrogen are selectively affected. The marked decline in nitrification implies that the nitrifiers are relatively intolerant. Kreutzer (1963) thought of them as being a very specialized group restricted to a few genera of bacteria, which could all be susceptible to sterilants. Conversely, the ammonifiers include a wide spectrum of micro-organisms, so that the increase in ammonification may mean that the ammonifiers are stimulated, or that they are at least not adversely affected. This results in an imbalance between ammonium N and nitrate N.

With the fungi, again there are different effects. Thus *Trichoderma hamatum* and *Chrysosporium pannorum* are relatively tolerant, while *T. viride* is relatively susceptible. Use of this fact can be made to assess the level of toxicity of the fungicide: by assessing the potential of *C. pannorum* and *T. viride*, as compared with a control soil, for example, these species can be used as indicators of the remaining toxicity.

When we came to inspect the overall effect of fungicides on the fungi in the soil, an initial reaction is that after about 6 weeks the fungi are returning to their pre-treatment levels. However, as will be apparent from the use of species as indicators, there are changes at the specific level; and repeated applications of a given fungicide can cause an alteration in the species composition which may not be apparent when fungal 'numbers' are counted. There will tend to be a limitation of fungal species, with the virtual elimination of those susceptible species which are not rapid recolonizers, and a concomitant increase in those which are tolerant as well as those which can rapidly invade the soil as the level of remaining toxicity falls to a tolerable level. Thus the community, taken as the totality of species living actively together at a given time, will change. Similarly, the population of individual species will also change, depending upon its degree of tolerance to the fungicide.

However, these changes in the active community and in the active population of individual species cannot be measured using the conventional, orthodox methods for isolating soil fungi. From numbers of isolations, whether made on dilution or soil crumb plates, we cannot even accept that a species becomes dominant after treatment. All we can say with certainty is that a given species becomes more frequently isolated and now forms a larger proportion of the fungal potential of that soil—because we do not know whether or not those extra isolations have resulted from an increased sporulation without any increase in overall metabolic activity.

The effects of the fungicides on the Basidiomycete population require further studies. It is apparent that the mycorrhizal species tested are less tolerant of the agrochemicals used than are the non-mycorrhizal species. We do not yet know the effects on the pathogenic, soil-borne Basidiomycetes. However, any adverse effects on the decomposing activities of the non-mycorrhizal species may disturb the recycling of nutrients in the soil, while the greater effects on

the mycorrhizal species could harm the growth of the higher plants with which they are associated. This could be especially important in the use of such agrochemicals in the upgrading of marginal lands, where many plants have mycorrhizal associations.

The preliminary studies on species of *Fusarium* indicate that normally useful species such as *F. culmorum* could be eliminated from soils by using dose rates which do not necessarily remove the pathogenic species.

At low rates of application, fungicides can help bring about increased growth of higher plants (Kuthubutheen, 1977). This can be caused by changes in the amino acid content of the soil following treatment (Wainwright and Pugh, 1975a) and the production of growth-promoting substances (Wainwright and Pugh, 1975b), as well as by a general increase in availability of nutrients from the activities of tolerant microbes on the dead fungal biomass, and possibly also through an increase in activity of microorganisms which are involved in mineralization and nutrient release. Thus the short-term effects of fungicide application can be beneficial to soil fertility (Wainwright, 1974). However, the long-term reduction in the potential of cellulose-decomposing fungi following fungicide treatment (Pugh and Williams, 1971) can have a marked effect on soil structure and properties. The accumulation of undecomposed plant remains at the soil surface can lead to waterlogging and conditions of greatly reduced aeration, which can further reduce general microbial growth, but which can stimulate the activities of anaerobic bacteria.

The need is apparent, therefore, to develop further the study of the effects of fungicides on soil organisms and on soil fertility. The concept of a dynamic equilibrium among soil microorganisms is valid under stable conditions. The application of fungicides, or of any other biocide, however, by affecting that stability, alters the conditions within the soil. There then follow several concomitant changes:

- (1) The fungal potential is reduced, both in terms of the fungal community, as measured by the range of species present, and in terms of the populations of individual less-tolerant species.
- (2) As the fungicide leaches away or is biodegraded, there is recolonization of the soil. Initially this is by the most tolerant of the susceptible species, and later by good competitors, which may have little or no tolerance of the fungicide. Patterns of change within the fungal potential will vary with different fungicides.
- (3) The application of fungicides thus enhances the importance of a few tolerant species. This new importance will remain as long as the balance is tilted in favour of those species; for example, by the use of a persistent fungicide or by repeated application of a non-persistent fungicide. The changes in the fungal potential can be used to plot the toxicity levels present.
- (4) The dynamic balance between fungi and bacteria is at first changed in favour of the bacteria, but later returns to the pre-treatment situation as fungi return.
- (5) The fungal potential may appear to return to the pre-treatment level quantitatively, but there will be qualitative changes which are more difficult to detect, as they require identification of fungi at the specific level.

- (6) The changes in the balance between fungi and bacteria can affect the various microbial processes in the soil, leading to changes in nitrogen turnover and a decrease in the rate of decomposition of plant remains with concomitant changes in soil structure.

However, these changes appear to be relatively short-lived, except where there are regular applications of fungicides with long-lasting residual effects, and especially when these are used in routine prophylactic applications.

While the development of new fungicides will proceed in the future, and their uses will expand as greater food production becomes ever more necessary, their effects on non-target organisms will need to be constantly monitored.

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PHYTOALEXINS

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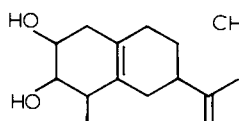
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W.G. RATHMELL

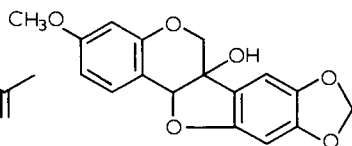
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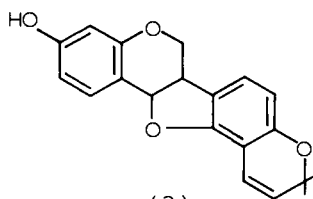
The concept of phytoalexins, which are now defined as 'low molecular weight antimicrobial compounds that are both synthesized by and accumulate in plants after their exposure to microorganisms', was first evoked by Müller and Börger (1940) to explain their experiments on the resistance of potatoes to different physiological races of the fungus *Phytophthora infestans*. It was found that if potato tuber slices were inoculated with an avirulent race of the fungus, to which the potato cultivar was resistant, the inoculated tissue became resistant to a second inoculation by a virulent race to which, under normal conditions, the tuber was susceptible. Müller and Börger postulated that the incompatible interaction induced the formation of a phytoalexin which inhibited the compatible race of the fungus. Although it took 28 years to determine that the major potato phytoalexin was the sesquiterpenoid rishitin (1), in the intervening period considerable progress was made into the nature of phytoalexins produced in other plant families, particularly in the Leguminosae. Here also Müller carried out the pioneering experiments when he inoculated French bean pods (*Phaseolus vulgaris*) with spore suspension of *Monilinia fructicola*, a fungus which is not a pathogen of beans (Müller, 1958). From similar experiments with pea pods (*Pisum sativum*) Cruickshank and Perrin (1960) isolated pisatin (2) (Perrin and Bottomley, 1962), and later the same group of workers isolated phaseollin (3) from bean pods (Cruickshank and Perrin, 1963; Perrin, 1964).



(1)
Rishitin

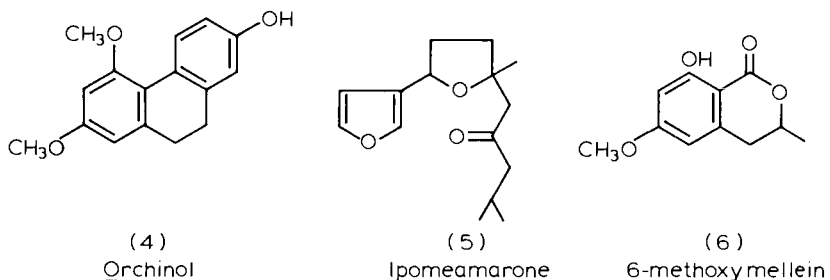


(2)
Pisatin



(3)
Phaseollin

In the intervening period, antifungal compounds were found in other plants. Orchinol (4), which was isolated by Gäumann and Kern (1959a; 1959b) from both apparently healthy tubers of *Orchis militaris* and from surface-sterilized segments inoculated with *Rhizoctonia repens*, was characterized by Hardegger, Biland and Corrodi (1963). Ipomeamarone (5) was identified by Kubota and Matsura (1953) as the antifungal compound which accumulated in roots of sweet potato, *Ipomoea batatas*, after infection by *Ceratocystis fimbriata*. Condon and Kuć (1960; 1962) isolated the substituted dihydroisocoumarin, 6-methoxymellein (6), from infected roots of carrot; the same compound was also isolated by Sondheimer (1961) from carrots stored at 0°C.

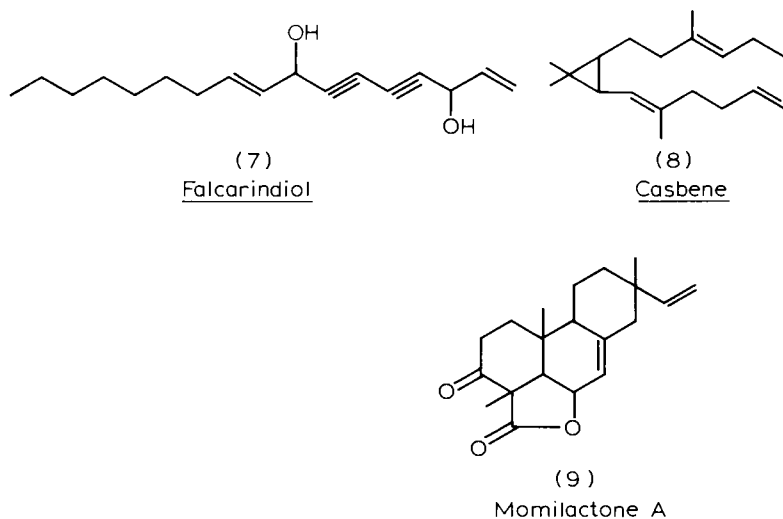


Since the experiments of Müller and Cruickshank, non-pathogens have been widely used to test whether or not plants will produce phytoalexins in response to a fungal challenge. In particular, non-pathogens have been used in the 'drop-diffusate' technique of Müller: drops of spore suspension are applied to the host tissue and, after incubation, the liquid is collected, centrifuged and tested for activity. It is possible to isolate phytoalexins such as pisatin from diffusates simply by extraction with immiscible solvents such as light petroleum. One advantage of the drop-diffusate technique is the absence of plant pigments, which might otherwise interfere with chromatographic separations. Cruickshank and Perrin (1961) proposed that the plant cell membrane is, in effect, a primary 'filter'.

This relatively simple extraction procedure is often followed with a combined chromatographic separation and bioassay on thin-layer chromatography plates, which are sprayed with spore suspensions of test organisms such as *Cladosporium cucumerinum* (Bailey, 1973). The use of these two techniques in screening has produced results which are perhaps of more use in chemotaxonomic surveys than in plant pathological investigations. In the latter it is important to extract tissues with organic solvents, since the phytoalexin concentration in the tissue itself may be different from that in drop diffusates (Khan and Milton, 1978).

Taxonomic implications of phytoalexin accumulation

Phytoalexin accumulation by plants appears to be a feature of taxonomic significance. The families which have been most widely examined are the Leguminosae and the Solanaceae. Six isoflavonoid classes predominate in the



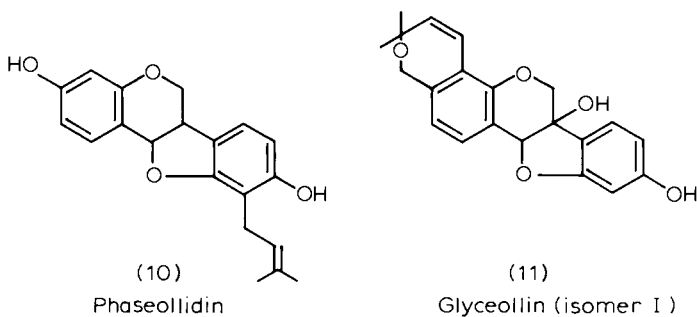
Leguminosae, and other chemical classes include the phenolic benzofurans, chromones, flavanones and stilbenes; the furanoacetylenes are found in species of *Vicia* and *Lens*. Phytoalexin accumulation has been used by Ingham and Harborne (1976) to divide the genus *Trigonella* into three major groups according to their ability to produce the pterocarpan medicarpin and maackiain and the isoflavans vestitol and sativan (see later). In the Solanaceae, the major compounds are the norsesqui- and sesqui-terpenoid compounds found in potato, peppers, tobacco and tomato (e.g. rishitin); the latter also produces polyacetylenic compounds like falcarindiol (7). Other families contain plants which accumulate phytoalexins of diverse chemical types. Chemically unusual compounds are the diterpenoid hydrocarbon casbene (8) isolated from infected castor bean seedlings (Sitton and West, 1975) and the 9- β -pimaradiene diterpenes, momilactone A (9) and momilactone B, found in leaves of rice infected by *Pyricularia oryzae* (Cartwright *et al.*, 1981).

Role in natural disease resistance

The results of different types of experiments have been used to support the hypothesis that phytoalexins are of importance in the protection of plants against (fungal) pathogens. These results point to four main features relating phytoalexin accumulation and metabolism to plant-pathogen interactions. First, phytoalexins rapidly accumulate to a fungitoxic level in resistant plants and there is either lesser or slower accumulation in susceptible plants. Secondly, pathogens are less susceptible to phytoalexins than non-pathogens. Thirdly, when the accumulation of phytoalexins is either increased or decreased by manipulation of the experimental conditions, the plants become either more resistant or more susceptible. Fourthly, pathogenic fungi can metabolize phytoalexins to less fungitoxic products. It is clear that all four features are not necessarily demonstrated in each of the host-pathogen interactions; this probably indicates that resistance of plants to pathogens involves physiological responses other than phytoalexin accumulation.

A range of host-pathogen combinations have been used in published experiments. Compatible and incompatible reactions of host-pathogen combinations which have been compared have often been obtained using several cultivars which give differential reactions to the same race of the pathogen. Alternatively, the same cultivar with two races of the pathogen, an avirulent one which gives a resistant reaction and a virulent one which gives a compatible reaction, have been used. Alternatively, different fungi which are pathogenic to different extents on the same host have been compared. Most of the interactions examined in detail for phytoalexin accumulation include host plants from among the Leguminosae and the Solanaceae.

The accumulation of isoflavonoid phytoalexins has been examined in a French bean cultivar which is resistant to race β of *Colletotrichum lindemuthianum* at both 17°C and 25°C and is susceptible to race γ at 17°C but resistant at 25°C. In the resistant reaction, there were high concentrations of phaseollin (and phaseollinisoflavan) but lower concentrations of phaseollidin (10) (and kievitone). The concentrations of all four compounds were lower in the susceptible infected plants (Bailey and Deverall, 1971; Bailey, 1974).



The phytoalexin glyceollin (11) accumulates at different rates in soya beans, depending upon the reaction of the cultivar to different races of *Phytophthora megasperma* f.sp. *glycinea*. The cultivar Harosoy is susceptible to race 1 of the fungus; Harosoy 63 is resistant to race 1, but susceptible to race 4. In two separate series of experiments, it was found that in the resistant reactions with Harosoy 63 and race 1, large amounts of glyceollin accumulated, but there were much lower levels in Harosoy/race 1 or in Harosoy 63/race 4 (Yoshikawa, Yamauchi and Masago, 1978; 1979).

There are a number of reports which indicate that, in general, phytopathogenic fungi are relatively insensitive to the phytoalexins produced by their respective hosts, whereas non-pathogens are sensitive (VanEtten and Pueppke, 1976; Smith, 1982).

However, there are exceptions to this generalization (Smith, 1982). The best known is the case of *Aphanomyces euteiches*, which is extremely sensitive to pisatin when tested *in vitro*. Nevertheless, the fungus grows extensively in pea tissues, even though the pisatin concentration in lesions may be as much as eight times that which gave complete inhibition of growth in the bioassay (Pueppke and VanEtten, 1974).

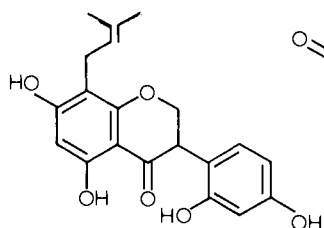
Conditions which decrease phytoalexin production are the treatment of Harosoy 63 soya beans with either blasticidin S or actinomycin D and treat-

ment with heat; in each case the plants become susceptible to race 1 of *P. megasperma* to which they are normally resistant (Yoshikawa, Yamauchi and Masago, 1978).

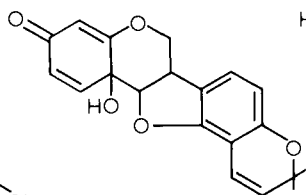
The resistance of soya bean and French bean to *P. megasperma* and *C. lindemuthianum*, respectively, can be induced by treatment of hypocotyls with u.v. radiation prior to inoculation; in each case it is assumed that phytoalexins accumulate in response to u.v. (Bridge and Klarman, 1973; Andebrhan and Wood, 1980).

Many fungi actively metabolize phytoalexins; it has been known for some time that pterocarpan phytoalexins such as phaseollin, pisatin and medicarpin disappear when they are added to actively growing cultures of fungi (Pierre and Bateman, 1967; de Wit-Elshove, 1968; Higgins and Millar, 1969). Metabolism of phytoalexins appears to be one of the major reasons for the greater pathogenicity of *Fusarium solani* f.sp. *phaseoli* compared with that of *Rhizoctonia solani* on French beans. Inoculation of hypocotyls with *F. solani* gives spreading lesions, whereas *R. solani* inoculation gives restricted lesions after penetration of hypocotyl tissue.

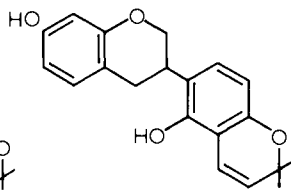
In *F. solani* infection, kievitone (12) was present in small quantities at 24 h but fell to only trace levels at 120 h, while phaseollin continued to accumulate up to 120 h after inoculation (Morris and Smith, 1978). Other compounds found were 1a-hydroxyphaseollone (13), a smaller amount of phaseollinisoflavan (14) and traces of 2'-*O*-methyl phaseollinisoflavan (15) and phaseollidin. 1a-Hydroxyphaseollone and 2'-*O*-methyl phaseollinisoflavan are fungal detoxification products of phaseollin and phaseollinisoflavan, respectively (Heuvel and VanEtten, 1973; VanEtten, 1973). This contrasts markedly with



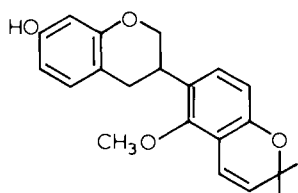
(12)
Kievitone



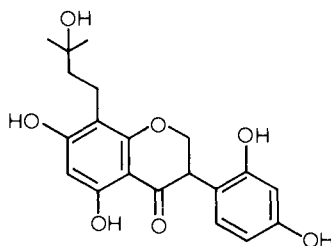
(13)
1a-Hydroxyphaseollone



(14)
Phaseollinisoflavan



(15)
2'-*O*-methyl phaseollinisoflavan

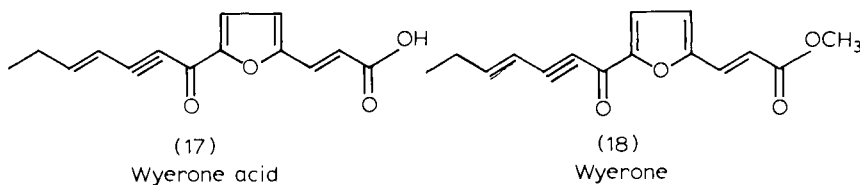


(16)
Kievitone hydrate

R. solani infections, where kievitone increased in amount from young to intermediate to mature lesions (96 h after inoculation); the phaseollin level was lower in both young and intermediate lesions but increased considerably in mature lesions (Smith, VanEtten and Bateman, 1975). It was concluded by these workers that the levels of phytoalexins, and particularly of kievitone, were sufficiently high to inhibit the mycelial growth associated with the limitation of the lesions. The metabolism of kievitone by *F. solani* has been demonstrated *in vitro*; when kievitone was added to a liquid medium in which *F. solani* was growing, kievitone disappeared and was replaced by kievitone hydrate (16) which is less fungitoxic (Kuhn and Smith, 1976; Kuhn, Smith and Ewing, 1977). An enzyme, kievitone hydratase, not only catalyses the hydration of kievitone to kievitone hydrate but also of phaseollidin to phaseollidin hydrate (Smith *et al.*, 1980).

Another factor which may explain the difference between the levels of phytoalexin accumulation in French beans following inoculation with *F. solani* and *R. solani*, is that of phytoalexin elicitation (see below). When cell-free extracts of the two fungi were compared for their ability to elicit phytoalexins in decored excised French bean hypocotyls, Morris and Smith (1978) found that, whereas the *R. solani* extracts would elicit high levels of kievitone, the *F. solani* extracts elicited only trace amounts.

Phytoalexin metabolism is an important facet of the pathogenicity of *Botrytis fabae* compared with *B. cinerea*, a non-pathogen on broad beans (Mansfield, 1982). Inoculation with *B. cinerea* causes the accumulation of large amounts of wyerone acid (17) and smaller amounts of wyerone (18);



fungal growth stops by 12 h. With *B. fabae* fungal growth continues at a steady rate for longer periods and only small amounts of the two phytoalexins are found. Since *B. fabae* can tolerate higher concentrations of wyerone and also appears to metabolize wyerone acid much faster than other *Botrytis* spp. (Rossall and Mansfield, 1978), Mansfield (1982) has accordingly suggested that the difference in phytoalexin accumulation in the response of broad beans to *B. fabae* and *B. cinerea* must be a difference in the balance between phytoalexin production and degradation in the two interactions.

Elicitation of the phytoalexin response

In many of the examples cited above, differential elicitation of phytoalexins by pathogens is an important determinant of phytoalexin accumulation. In experiments designed to determine the mechanism of elicitation, many attempts have been made to isolate so-called elicitors from pathogenic and non-pathogenic fungi. One of the first elicitors to be isolated was monilicolin

A, obtained from culture filtrates of *Monilinia fructicola* which elicited phaseollin accumulation in French bean pods and was characterized as a polypeptide (Cruickshank and Perrin, 1968). Most of the recent work has been on carbohydrate-containing elicitors obtained from mycelium and culture filtrates of *Colletotrichum lindemuthianum* and *Phytophthora megasperma* f.sp. *glycinea* which elicit phytoalexin accumulation in French bean and soya bean, respectively.

Anderson (1978) obtained oligosaccharides from culture filtrates of a race of *C. lindemuthianum* and from two non-pathogens of French bean, namely *C. trifolii* and *C. destructivum*. The most active fraction from all three fungi contained mainly glucose and small amounts of galactose; small amounts of mannose and rhamnose were also found in the elicitors from the two non-pathogens. Activity of all three was lost after periodate oxidation and after treatment with an endoglucanase.

The extensive investigations carried out by Albersheim and his co-workers on the elicitors from *P. megasperma* which elicit both cell browning and the accumulation of glyceollin in soya beans have been summarized by Albersheim and Valent (1978). Elicitors which have been obtained from culture filtrates or by heat treatment of cell walls are similar in structure and biological activity; the latter have been examined in more detail. The active moieties appear to be β -1,3 and β -1,6 glucans. Both the yeast and *P. megasperma* elicitors will also elicit the accumulation of phenolic phytoalexins in red kidney beans and of terpenoid phytoalexins in the potato. There was no indication of any race-specificity in the preparations. It appears that the soluble phytoalexin elicitor, which Yoshikawa, Matama and Masago (1981) found was released after incubation of *P. megasperma* cell walls with soya bean tissue, has very similar properties to the highly purified elicitor isolated by Albersheim and colleagues. There was an indication that race-specificity in the *P. megasperma* soya bean system is controlled by extracellular glycoproteins (ECGP) of the fungus (Wade and Albersheim, 1979). ECGPs from incompatible races, when introduced into hypocotyl wounds, protected the seedlings against a fungus inoculum from a compatible race. However, a recent publication from the same laboratory (Desjardins *et al.*, 1982) reports that there was considerable variation in the ability of ECGP to protect seedlings from infection. Bioassay variation was observed with all fungal races, all preparations of ECGP and all soya bean cultivars tested. This variability in the assay has effectively blocked any further investigations on the purification and characterization of the factors involved in the protection reactions. Ziegler and Pontzen (1982) claim that extracellular invertases from *P. megasperma*, which are mannan-glycoproteins, can act as race-specific suppressors of phytoalexin accumulation in soya bean. The carbohydrate moieties of the extracellular invertases of three races of *P. megasperma* have structures unique to each race (Ziegler and Albersheim, 1977). Ziegler and Pontzen (1982) have also proposed that race-specific glycoproteins, similar to the extracellular invertases, might be present in *P. megasperma* cell walls; the presence of these specific inhibitors, together with non-specific elicitors, could explain the earlier claim of Keen and Legrand (1980) that cell wall glycoproteins can act as race-specific elicitors of glyceollin accumulation in soya beans.

Race-specific suppressors of phytoalexin accumulation in potato have also

been demonstrated in *P. infestans* (Doke and Tomiyama, 1980; Doke, Garas and Kuć, 1980). These compounds, assumed to be water-soluble glucans, will specifically suppress the eliciting action of either a non-specific hyphal wall elicitor or of incompatible races of the fungus. Many of the explanations proffered to explain the interactions between non-specific elicitors and race-specific suppressors imply that the elicitor and the suppressor are competing for specific sites on a receptor in the host plant. Such an explanation might be too simple for the potato—*P. infestans* interaction, where it now appears possible that either arachidonic or eicosapentaenoic acids may be active as non-specific fungal elicitors (Bostock, Kuć and Laine, 1981). It would be necessary to postulate that the fatty acids alter the molecular orientation of host cell membranes, which may also contain a receptor for carbohydrate elicitors and/or suppressors.

Elicitation by other treatments

A wide range of chemicals, especially the salts of such heavy metals as mercury and copper, elicit the accumulation of phytoalexins in different families. Examples are given in *Table 7.1*.

It has been mentioned that phytoalexin accumulation may also be triggered by u.v. light treatment. Damage to host tissue by freezing and thawing causes the accumulation of isoflavonoid phytoalexins, particularly phaseollin, in French beans (Hargreaves and Bailey, 1978). Furthermore, aqueous extracts of damaged bean tissue stimulated the production of phaseollin in hypocotyl tissue and a range of phytoalexins in cell suspension cultures of *Phaseolus vulgaris* (Hargreaves and Selby, 1978). Exudates from cotyledons of both bean and pea treated with mercuric chloride were able to stimulate phyto-

Table 7.1 ELICITING EFFECTS OF HEAVY METAL SALTS AND SURFACTANTS

<i>Elicitor</i>	<i>Plant</i>	<i>Phytoalexin</i>	<i>Reference</i>
HgCl ₂	Pea	Pisatin	Perrin and Cruickshank (1965)
	French bean	Phaseollin	Hargreaves (1979); Rathmell and Bendall (1971)
	Soya bean	Glyceollin	Yoshikawa (1978)
	Broad bean	Wyerone and related compounds	Mansfield, Porter and Smallman (1980)
	Carrot	6-Methoxymellein	Kuć (1972)
	Sweet potato	Ipomeamarone	Uritani, Uritani and Yamada (1960)
	Potato	Rishitin	Cheema and Haard (1978)
CuCl ₂	Pea	Pisatin	Perrin and Cruickshank (1965)
	Soya bean	Glyceollin	Klarman and Sanford (1968)
	Carrot	6-Methoxymellein	Kuć (1972)
Triton-X-35	French bean	Phaseollin	Hargreaves (1981)
Triton-X-100	Soya bean	Glyceollin	Yoshikawa (1978)

alexin accumulation in cotyledons of the same species (Hargreaves, 1979). These experiments imply that host cell damage causes the release of host elicitors of phytoalexin accumulation; they have been termed constitutive elicitors. Very similar effects have been obtained by Hahn, Darvill and Albersheim (1981) using a soya bean system; hot water extracts of either soya bean tissue or cell walls or acid hydrolysate of cell wall of soya bean, tobacco, sycamore and wheat yielded what were termed endogenous elicitors. The endogenous elicitors caused the accumulation of glyceollin in soya bean cotyledons. More recently, Lyon and Albersheim (1982) have found that an extract of frozen and thawed soya bean stems is a heat-labile elicitor of glyceollin, but that it releases heat-stable elicitor-active molecules from soya bean cell walls. It is suggested that the heat-stable elicitor might be similar to the endogenous elicitor described by Hahn and co-workers, although a direct comparison has not been made. Lyon and Albersheim (1982) also raise the possibility that the heat-labile elicitor may be involved in the accumulation of phytoalexins when cells are damaged by u.v. light, heavy metals or detergents.

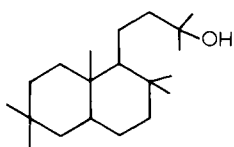
Other manifestations of resistance

It was indicated earlier that phytoalexin accumulation is not the only physiological response involved in the resistance of plants to pathogens. The accumulation of insoluble phenolic compounds in the lignification and suberization processes is also important (Friend, 1981). These responses, like phytoalexin accumulation, are involved in the reactions of non-hosts to pathogens (non-specific resistance) or of resistant hosts to avirulent races of pathogens (race-specific resistance). It seems likely that the recognition processes underlying all three types of physiological response are similar (Friend, 1981). It is also possible that a plant may use a combination of all three responses in its resistance mechanism. The relative importance of each one may vary between different cultivars of the same host and possibly with the physiological age of the host. Kuć (1982) cites the example of young potato tubers harvested early in the growing season. These showed the expected compatible and incompatible reaction to appropriate races of *Phytophthora infestans*. Nevertheless there appeared to be no difference in the timing or amount of phytoalexin accumulation between the incompatible and compatible interactions. Kuć accordingly suggested that, in young tubers harvested in the field, either lignification or suberization rather than phytoalexin accumulation may be the factor limiting fungal development.

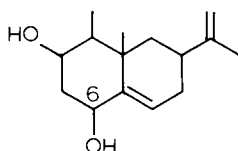
Phytoalexins as fungicides

It has been suggested that phytoalexins and other antifungal compounds from plants may be sufficiently toxic to fungi to be used as conventional fungicides. Bailey *et al.* (1975) found that the diterpene sclareol (19), obtained from tobacco (*Nicotiana glutinosa*) leaf-surface exudates, would control rust (*Uromyces appendiculatus*) infections of French bean when sprayed onto the plant's foliage at concentrations of 100 µg/ml. Similarly, Ward, Unwin and Stoessl (1975a) found that 5×10^{-4} mol/l (118 µg/ml) solutions of capsidiol

(20) reduced by about 90% the number of lesions of *P. infestans* that developed on tomato plants in a growth room. At higher concentrations, this level of disease control persisted for up to 8 days.

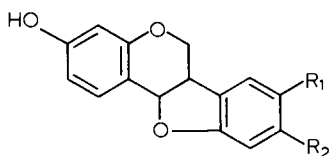


(19)
Sclareol



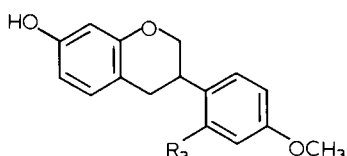
(20)
Capsidiol

On the other hand, Rathmell and Smith (1980) were unable to detect useful protectant fungicide activity in a range of isoflavonoid phytoalexins when they were tested at similar application rates (up to 100 $\mu\text{g/ml}$) against six different pathogens. The compounds tested included kievitone, medicarpin (21), maackiain (22), vestitol (23) and sativan (24).



(21)
 $R_1 = \text{H}; R_2 = \text{OCH}_3$ Medicarpin
(22)

$R_1 + R_2 = \text{OCH}_2\text{O}$ Maackiain

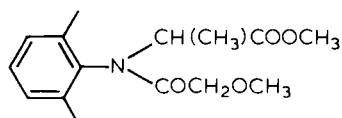


(23)
 $R_3 = \text{OH}$ Vestitol
(24)

$R_3 = \text{OCH}_3$ Sativan

These are the only experiments known to the writers in which phytoalexins and similar compounds have been assayed for use as fungicides on whole test plants rather than *in vitro*. In all the experiments the observed levels of antifungal activity were lower than those that can be achieved with synthetic compounds in similar bioassays. The synthetic acylanilide fungicide metalaxyl (25) almost completely inhibits the sporulation of *P. infestans* on potato foliage at rates of spray application as low as 1–2.5 $\mu\text{g/ml}$ (Bruck, Fry and Apple, 1980). This is some 40-fold greater activity than that of capsidiol in the work of Ward, Unwin and Stoessl (1975a).

Hence, the limited experimental evidence available so far suggests that phytoalexins are only weakly active as conventional fungicides. If phytoalex-



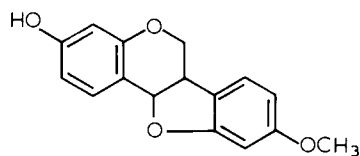
(25)
Metalaxyl

ins play any role in natural disease resistance, localized accumulation near the site of infection appears to be an important part of the mechanism (Deverall, 1977) and this cannot easily be achieved by artificial application. However, the results obtained so far do not rule out the possibility that phytoalexins that have a much higher level of fungicidal activity will be characterized in the future. As a group, the terpenoids such as the momilactones A and B appear more active than other types of phytoalexin, as judged by ED_{50} values *in vitro* (Cartwright *et al.*, 1981).

Phytoalexins as models in a programme of fungicide synthesis

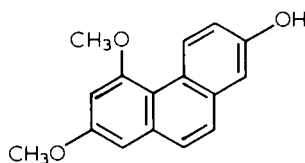
Total synthesis of phytoalexins has often been achieved, frequently as a part of structure and/or biosynthesis studies. Maackiain used in the work of Rathmell and Smith (1980; see previous section) was obtained in racemic form by chemical synthesis (Dewick, 1975).

It appears very unlikely, however, that synthetic programmes based on phytoalexins will be attractive to pesticide chemists. First, there is very little information as to which of the structural features of the phytoalexins are important to their activity. For example, the study of pterocarpan and isoflavonoid compounds by VanEtten (1976) failed to support an earlier hypothesis that a particular stereochemical conformation was required for antifungal activity. Moreover, both the + and – enantiomers of 3-hydroxy-9-methoxypterocarpan (26) were found to be equally active against *Aphanomyces euteiches* and *Fusarium solani* when tested *in vitro*. This suggests either that there is no specific receptor in the fungal cells for the compound or that the enantiomers have different modes of action with different receptor sites. VanEtten suggests—from a study of data from other compounds—that a common physicochemical basis for the antifungal activity of the various isoflavonoids may not exist.



(26)

3-hydroxy-9-methoxypterocarpan



(27)

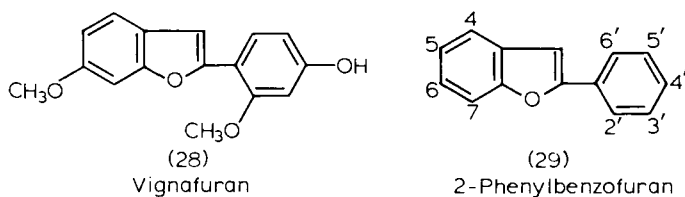
Dehydroorchinol

A similar conclusion can be reached from the data of Ward, Unwin and Stoessl (1974) on a series of sesquiterpenes related to capsidiol. These workers found that, for a range of fungi, relative inhibition by capsidiol of spore germination and mycelial growth *in vitro* were not correlated. Certain structural features of the molecule—such as the configuration of the 6-hydroxyl—appeared critical for activity, whereas others—such as the presence of the double bond in the side chain—did not. However, oxidation of the 6-hydroxyl to a ketone caused loss of activity against spore germination of some fungi (e.g. *Monilinia fructicola*) yet not others (e.g. *Phytophthora capsici*). It caused only partial loss of activity against mycelial growth. Some compounds lacking

the 6-hydroxyl were, however, active, although none to the same extent as capsidiol. It was not possible to make unambiguous conclusions about structure-activity relations from this work.

Ward, Unwin and Stoessl (1975b) examined stilbenes and phenanthrenes related to phytoalexins and again found difficulties in reaching conclusions about structure-activity relations. Some of the levels of activity *in vitro* recorded were quite high, however. The ED_{50} for dehydroorchinol (27) against *P. infestans* was recorded as 5×10^{-6} mol/l (1.25 μ g/ml).

Carter, Chamberlain and Wain (1978) found that a series of molecules related to viginafuran (28) (a phytoalexin from *Vigna unguiculata*) also showed different relative activity depending on whether spore germination or growth inhibition was measured. The unsubstituted 2-phenylbenzofuran (29) was inactive, but hydroxylation at the 6,7,2' or 3' positions gave active compounds. Hydroxylation at the 5 position did not give activity; hydroxylation at the 4' position gave good activity (better than viginafuran) against some fungi, but none against others in spore germination tests. Addition of more



hydroxyl groups decreased activity, perhaps because lipophilicity was decreased (measured by R_F on reversed-phase TLC). Provided at least one hydroxyl group (at an active position) was present in the molecule, an additional methoxy group sometimes increased its activity, perhaps because the lipophilicity was restored to the correct range. Thus, vignafuran and 2-(2'-methoxy-4'-hydroxyphenyl)-benzofuran were more active than 2-(4'-hydroxyphenyl)-benzofuran in the spore-germination test. Vignafuran was not the most active compound in this test (2-(2'-hydroxyphenyl)-benzofuran was best followed by its 4'-methoxy analogue). When the analogues were tested *in vivo* as foliage sprays, however, at 100 μ g/ml, against several diseases they gave mediocre control (generally less than 30% disease reduction) when compared with synthetic fungicides. This suggests that it may prove difficult sufficiently to improve the activity of phytoalexins by synthesis to make them commercially useful, even where some structure-activity patterns are evident.

There is a second reason why phytoalexins are unattractive as models in a programme of fungicide synthesis, which is that there is little reason to suppose that, even if sufficiently active compounds were made, they would be superior to compounds that are wholly synthetic in conception. For example, they would be just as likely to be injurious to non-target organisms (crop plants, wild life, consumers) as are the synthetic compounds that are in use today. Skipp, Selby and Bailey (1977) found that 10-min exposure of French bean cell-suspension cultures to phaseollin (50 μ g/ml) resulted in the death of more than 90% of the cells. Rather lower concentrations than this affected growth and respiration. Thus, the plant that produces phaseollin is scarcely less sensitive to it than are fungi.

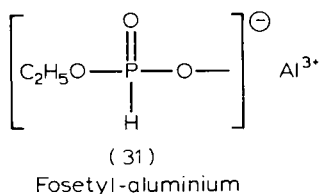
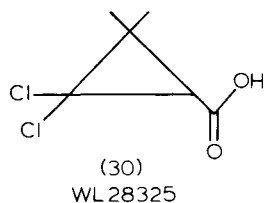
The same compound is toxic to water snails (*Australorbis glabratus*) and other invertebrates at concentrations of 20 µg/ml or less (Bailey and Skipp, 1978). The oral LD₅₀ values in mice of ipomeamarone and related compounds range between 38 and 230 mg/kg body weight (Boyd *et al.*, 1973, and references therein). This phytoalexin is therefore more highly toxic to mammals in acute tests than are most recently introduced synthetic fungicides.

Nor can it be assumed that phytoalexins would be less liable to the effects of decreased sensitivity in the population of the target fungus. Resistance to synthetic fungicides is a major problem in chemical disease control (see, for example, Davidse *et al.*, 1981). It is believed that fungicides with multiple modes of action may be less susceptible to the problem of resistance than are site-specific compounds (Delp, 1980). It is generally felt that phytoalexins are multi-site toxicants (Smith, 1982). Whether or not this generalization is true, there is evidence that *Colletotrichum lindemuthianum*, for example, can adapt to increasing concentrations of phaseollin (Skipp and Carter, 1978). The adaptation, or reduced sensitivity, was found to be a function of the time and extent of the pre-treatment of the fungus to the toxicant. Similar effects were observed with *Septoria nodorum* and with other isoflavonoid phytoalexins. It has already been mentioned that numerous fungi can metabolize phytoalexins to products that are usually, but not always, less toxic to them (VanEtten, Matthews and Smith, 1982).

In conclusion, there is little evidence that synthesis of analogues of the phytoalexins known at present will give rise to fungicides with any novel features. The studies already undertaken show that such compounds lack sufficient activity and selectivity and there are no signs of novel features. New discoveries of phytoalexins may change this situation; however, the phytoalexins are poor models for fungicide chemists at present.

Use of the phytoalexin response for practical disease control

Several workers have suggested that the phytoalexin response might be used for practical disease control, through the application of some form of triggering agent, either to induce increases in natural phytoalexin levels, or to potentiate the natural response to infection. Langcake's group (Cartwright, Langcake and Ride, 1980) have studied extensively the response of rice plants infected with blast (*Pyricularia oryzae*) to the experimental fungicide WL 28325 (2,2-dichloro-3,3-dimethyl-cyclopropane carboxylic acid) (30). This substance greatly increases the quantity of the momilactone phytoalexins that are produced at about the time that fungal development is checked. Uninfected plants produce negligible amounts of phytoalexin. It is evident from



the work of Cartwright and co-workers that this potentiation could be the mode of action of WL 28325.

Whether potentiation is achieved by a direct effect on the metabolism of the infected plant, or via an effect on the fungus, which in turn releases elicitors into the plant, is unclear. It has been known for some time that fungitoxic substances may change compatible host-fungus interactions into incompatible ones (Kiraly, Barna and Ersek, 1972). This is believed to be caused by the release of substances from the fungus following treatment with the fungitoxicant. Such release might occur without the growth of the fungus being affected, in some circumstances (Ward *et al.*, 1980). WL 28325 has low fungitoxicity *in vitro*. Inhibition of fungal growth might therefore be caused by an accumulation of phytoalexins that was triggered by the release of fungal elicitor. The techniques presently available do not enable us to distinguish between the various possibilities. It cannot therefore be said with certainty that WL 28325 represents an example of a fungicide that works by activating the phytoalexin response directly.

Similar difficulties arise in the interpretation of results being obtained by Bomeix and co-workers with fosetyl-aluminium (aluminium tris[ethyl phosphonate]) (31), a fungicide that is used in practical agriculture for the control of downy mildew (*Plasmopara viticola*) in vineyards (Chalandon *et al.*, 1979).

Again, it is found that detached leaves of tomato contain about 50% more phenols following infection with *Phytophthora capsici* and simultaneous treatment with fosetyl-aluminium than when only infected with the fungus and not treated with fungicide. Treatment with fungicides alone has no effect on the levels of phenols. It is assumed that the various phenolic compounds contribute to the inhibition of the growth of the fungus, because they are found to be inhibitory to a number of facultative pathogens *in vitro* (Raynal, Ravise and Bomeix, 1980). The physiological and cytological responses that result from simultaneous infection and treatment with fosetyl-aluminium resemble the natural defence reactions of plants, and this is taken as evidence for a direct effect of the fungicide (or a breakdown product, phosphorous acid) on the plant. It stops short, however, of absolute proof and direct effect on the fungus cannot be ruled out, although it is beginning to appear unlikely.

The response of susceptible plants treated with fungicides which, unlike fosetyl-aluminium and WL 28325, have a high level of intrinsic fungitoxicity, sometimes resembles the response of genetically resistant plants. Thus, Lazarovits and Ward (1982) observed that metalaxyl-treated soya bean plants produced the phytoalexin glyceollin when inoculated with a normally compatible race of *P. megasperma*. Without fungicide, very little glyceollin was produced. At a low dose of metalaxyl that did not inhibit development of the fungus, glyceollin was nevertheless produced to a concentration similar to that found with higher levels of fungicide. The authors concluded that it was metalaxyl, rather than glyceollin, that was decisive in preventing development of the pathogen, despite the apparent activation of the resistance response by the fungicide.

There are several other examples of work in which fungicides have been found capable of activating the phytoalexin response. In each case the evidence that this contributes to the fungicide's mode of action is slight. The intrinsic activity against the fungal mycelium is usually sufficient to account for the practical effectiveness of the compound. The only exception to this is

fosetyl-aluminium, where the preliminary evidence, summarized above, suggests that the compound works through the mediation of the plant's metabolism.

If it is assumed, for the time being, that fosetyl-aluminium has this unusual mode of action, the question arises whether this property has conferred any advantages on the fungicide over other conventional materials. Fosetyl-aluminium is an advanced fungicide in some respects, as it is able to move within the plant from sprayed foliage to new growth, being phloem-mobile. This reduces the need for the farmer to make repeated fungicide applications in the vineyard to obtain disease control throughout the season. It is not clear, however, that this property is necessarily linked to the compound's unusual mode of action.

In other respects, fosetyl-aluminium is less advanced. It does not have the very high levels of activity associated with modern fungicides (Rathmell and Skidmore, 1982). Thus fosetyl-aluminium is used in the field at twice the rate of the old non-systemic compound folpet, with which it is often co-formulated (Chalandon *et al.*, 1979).

The evidence available at present, therefore, does not suggest that fungicide technology will be advanced by compounds that activate the phytoalexin response. Our knowledge of the biochemical mechanism that triggers the response is, moreover, too slight to enable pesticide chemists to look for molecules that act in this way.

Elicitors themselves, or substances that mimic the action of fungal determinants of avirulence, might be considered as possible fungicides. Under controlled laboratory conditions elicitor from *P. megasperma* was able to protect soya bean hypocotyls from virulent races of the fungus (Albersheim and Valent, 1978). Schönbeck, Dehne and Balder (1982) protected grape, cucumber and wheat from their powdery mildews by applications of culture filtrates of fungi and bacteria under practical conditions. Tjamos (1982) obtained protection of cucurbits from a *Verticillium* sp. by foliage application of an elicitor isolated from the fungus. Sinha and Giri (1979) protected rice plants from blast (*Pyricularia oryzae*) by foliage or seed application of, for example, mercuric chloride (10^{-4} – 10^{-5} mol/l). Some control was obtained 5 weeks after application in a field experiment in the latter work. Although some of these experiments appear to have given promising results, there is little evidence at present that the disease control has been achieved by triggering a natural resistance response of the host plant in the same way that it might be triggered by a pathogen. Evidence of a specific recognition site on the plant would be needed to establish this, and such evidence is in most cases lacking. However, detailed biochemical work upon the action of elicitors is beginning to shed light on the mechanisms that may be involved (Hahlbrock *et al.*, 1981). Synthesis of m-RNA coding for enzymes of secondary metabolism can occur very rapidly following elicitor treatment of suspension cultured parsley cells. This is the first indication that elicitor action may be direct, rather than a result of general toxicity to plant cells.

In principle, the high sensitivity of specific plants to physiologically important elicitors of their defence reactions might give opportunities to develop extremely active chemicals as crop protection agents. Such substances would have a high degree of specificity of action and could overcome some of the important problems associated with the use of conventional fungicides. The

majority of the substances so far studied that appear to activate components of plants' defence reactions do not appear to act as a result of specific recognition by the plant. This is certainly true of unnatural compounds such as mercuric chloride. It also appears that a range of compounds of different types can trigger responses in plants which resemble the responses that occur when plants are incompatible with fungi (Rathmell, 1983). Pesticides based upon this mode of action would not necessarily have any novel features, such as high activity or specificity, that would help to advance chemical control technology.

The important steps that will be required to make further progress will be the elucidation of the nature of an elicitor-receptor site and further evidence as to the chemical nature of the physiologically important elicitors in a number of diseases.

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GIBBERELLIN SYNTHESIS INHIBITION BY HERBICIDES

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Introduction

The application of 'salt and ashes' by the Athenians to the site of Carthage after the fall of that city-state is an example of the use of 'toxicants' to prevent the growth of vegetation through soil sterilization. This treatment was 'non-selective' because it effectively prevents the growth of virtually all vegetation for as long as a toxic concentration exists. Prior to 1940, most 'chemical herbicides' available for commercial use were non-selective in nature and what 'selectivity' existed was due to differences in plant morphology and phenology. When the phenoxyalkyl herbicides were discovered (1940-45) they were recognized as selective antibiotics and utilized agriculturally very quickly; they are still in common use. However, after 40 years' experience and a vast literature on the subject, one of the latest statements on the activity of the phenoxy is that 'the plants become sick and die'. Since 1950, multitudes of chemicals have been synthesized and tested against a wide variety of species, and several of these compounds have found commercial usage as selective antibiotics.

More recently (Moreland, 1980), the accepted 'modes of activity' for herbicides were limited to: (a) photosynthetic inhibitors, (b) respiratory inhibitors, (c) membrane interactions and (d) cell division, nucleic acid metabolism and protein synthesis. Except for photosynthetic and respiratory inhibitors, there was not much definitive information available on the way various herbicides influence plant growth. This is due, partially, to the complexities inherent to plant biochemistry and the lack of basic information concerning plant processes other than photosynthesis and respiration. It is interesting to note that the major advances in understanding photosynthesis were made possible by the discovery of photosynthetic inhibitors originally utilized as herbicides (i.e. the phenylureas).

During the 1970s, the basic plant biochemistry of the synthesis of gibberellins (GA) was established in a series of excellent papers by West and co-workers (see Coolbaugh, Swanson and West, 1982) who used 'half-mature' wild cucumber seeds from Lower California as an enzyme source. This general synthetic pathway has now been extended to a few other tissues (see Hedden and Graebe, 1982), and it might be productive to use this general synthetic system to evaluate the activity of various herbicides as inhibitors of gibberellin precursor synthesis. The references listed herein constitute a very small por-

tion of the available literature on GA precursor biosynthesis and GA metabolism. Space limitations decreed the deletion of discussion of other work.

Gibberellin synthesis pathway

The carbon pathway for GA synthesis is through isoprenoid synthesis to geranylgeranylpyrophosphate (GGPP) (*Figure 8.1*). Several plant consti-

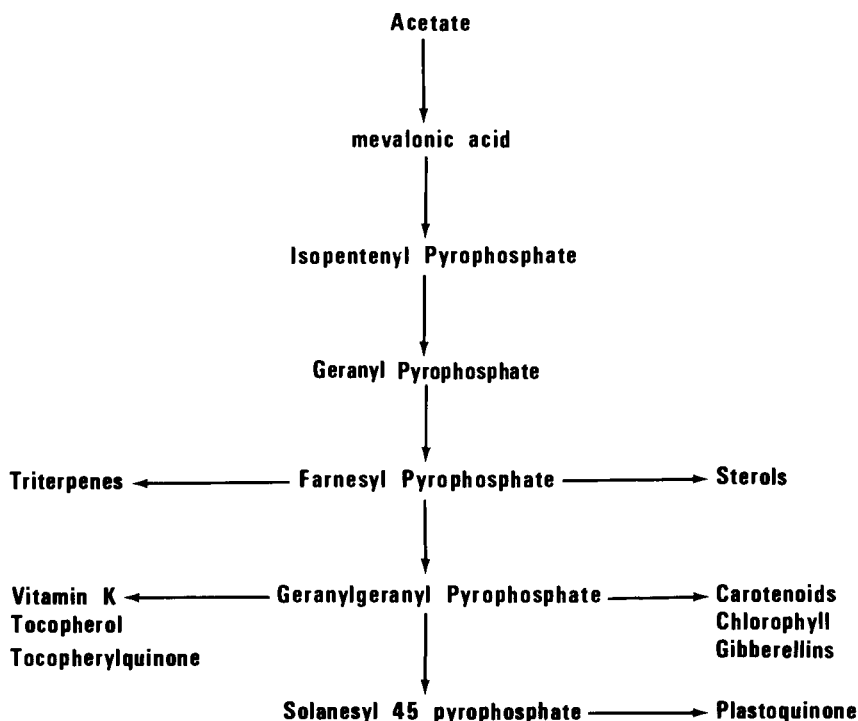


Figure 8.1 Carbon pathway in terpenoid synthesis

tents are produced by this enzymatic synthesis path and inhibition of the synthesis of one plant product quite often results in the accumulation of precursor and increased synthesis of another product which utilizes the same precursor(s). From GGPP, *ent*-kaurene is produced by cyclization (i.e. kaurene synthesis) and then oxidized (i.e. kaurene oxidation) to GA, after which GA metabolism synthesizes multiple GAs (*Figure 8.2*).

Chemicals

Chemical structures are shown in *Figure 8.3*. Trivial and chemical names are listed in *Table 8.1*.

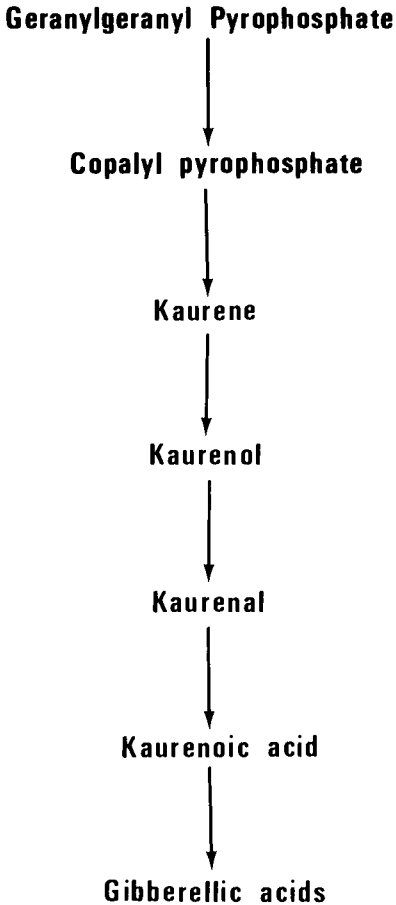
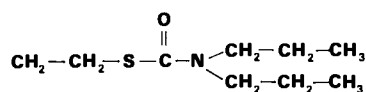
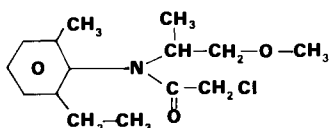
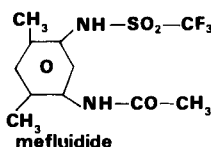
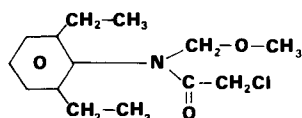
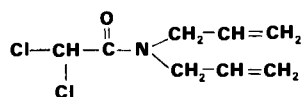
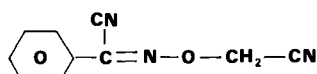
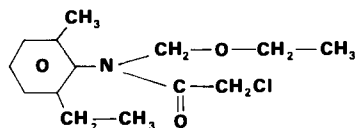
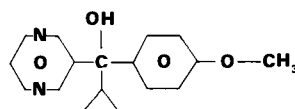
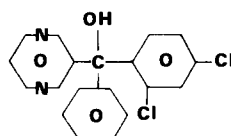
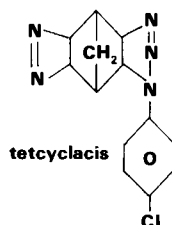


Figure 8.2 Biosynthetic pathway of gibberellic acid precursors

Growth responses

Plant growth inhibition responses are influenced by age or physiological stage of subject tissue, the method of application, plant species, antibiotic concentration, toxicant volatility, antibiotic degradation or metabolism and antibiotic chemical structure (*Table 8.2*). For example, EPTC is utilized in maize culture and is very effective as a selective herbicide. However, due to volatility problems, EPTC must be preplant-incorporated (PPI) into the soil prior to the planting of the crop. Extended season-long weed control requires 6.72 kg/ha, but this concentration is highly deleterious to the crop. Addition of 0.56 kg/ha R-25788 counteracts (i.e. antidotes or safens) EPTC in maize, but not in most weeds. Some maize cultivars are completely resistant to EPTC (6.72 kg/ha) without the addition of R-25788, whereas other cultivars are so sensitive to EPTC that R-25788 is not sufficiently effective to be of field use.

Similar considerations of stage growth, method of application, etc., apply

**EPTC****metolachlor****mefluidide****alachlor****R-25788****CGA-43089****acetochlor****ancymidol****triarimol****tetcyclacis****Figure 8.3** Chemical structures and trivial names of compounds**Table 8.1** TRIVIAL AND CHEMICAL NAMES OF COMPOUNDS

EPTC	<i>S</i> -ethyl dipropylthiocarbamate
Metolachlor	2-chloro- <i>N</i> -(2-ethyl-6-methylphenyl)- <i>N</i> -(2 methoxy-1-methylethyl) acetamide
Mefluidide	<i>N</i> -[2,4-dimethyl-5[[[(trifluoromethyl)sulfonyl]amino]phenyl]acetamide
Alachlor	2-chloro-2',6'-diethyl- <i>N</i> -(methoxymethyl)acetanilide
R-25788	<i>N,N</i> -diallyl-2,2-dichloroacetamide
CGA-43089	α -[(cyanomethoxy)-imino]-benzene acetonitrile
Acetochlor (Mon 097)	2-chloro- <i>N</i> -(ethoxymethyl)-6'-ethyl- <i>o</i> -acetotoluidide
Ancymidol	α -cyclopropyl- α -(4-methoxyphenyl)- α -pyrimidine methyl alcohol
Triarimol	α -(2,4-dichlorophenyl)- α -phenyl- α -pyrimidine methyl alcohol
Tetcyclacis	5-(4-chlorophenyl)-3,4,5,9,10-pentaaza-tetracyclo[5.4.1.0 ^{2,0} .1 ¹]dodeca-3,9-diene
Vernolate	<i>S</i> -propyl dipropylthiocarbamate

Table 8.2 CONCENTRATIONS OF COMPOUNDS REQUIRED FOR APPROXIMATELY 50% GROWTH INHIBITIONS AND THE APPLICATION TECHNIQUES REQUISITE TO THE ESTABLISHMENT OF ACTIVITY

Chemical	Plant species	50% growth inhibition	Application	Reference
EPTC	<i>Zea mays</i> (maize)	3.4 kg/ha (~ 18 mol/l) ^b	PPI ^a	Anon. (1979)
Metolachlor	<i>Sorghum bicolor</i> (sorghum)	0.5 p.p.m.w. (~ 1.8 mol/l)	PPI	Wilkinson (1981a)
Mefluidide	<i>Sorghum bicolor</i> (sorghum)	0.5 kg/ha (~ 1.6 µmol/l)	Post	Wilkinson (1982b)
Alachlor	<i>Sorghum bicolor</i> (sorghum)	0.14 p.p.m.w. (~ 0.5 µmol/l)	PPI	Wilkinson (1982a)
Ancymidol	<i>Pisum sativum</i> (English peas)	10 µmol/l	Post	Coolbaugh, Swanson and West (1982)
Tetacyclacis	<i>Oryza sativa</i> (rice)	500–1500 p.p.m.w. (~ 1.5 g/ha)	Seed Soak	Rieber and Jung (1982)

^aPPI, preplant incorporated; PE, pre-emergence; Post, post-emergence.

^bApproximate for comparison only.

to metolachlor, alachlor and mefluidide. Thus, some measure of the concentration of the toxicant in the tissue must be attained prior to conversion of field data to laboratory experiments. Vernolate is an analogue of EPTC (I) and their activity in wheat (*Triticum aestivum* L. cv. Holley) is virtually identical. At 125 p.p.b.w. (a.i.) vernolate incorporated into sand, the unruptured coleoptiles extend to about 1 cm above the sand surface and then growth ceases. When ¹⁴C-vernolate is incorporated into the sand at 125 p.p.b.w. (~3.3 µmol/l in the water in the sand) the coleoptiles contain ~1.8 µmol/l ¹⁴C-vernolate after 96 h when growth has stopped. This is based on the assumptions: (a) that the increase in fresh weight in the coleoptile is due to water absorption and ignores any possible metabolic conversions which would result in an increased fresh weight, and (b) that the ¹⁴C present is vernolate and not some degradation product. But the *maximum* vernolate concentration to which the seedling could be exposed is ~1.8 µmol/l. Since 1.12 kg active ingredient (a.i.)/ha (soil surface) is roughly equivalent to 1 p.p.m.w. in a soil layer ~15 cm deep, a field rate of 1 p.p.m.w. PPI would be about 8 × the absorption rate for vernolate. Therefore, the herbicidal activity of these materials lies, approximately, within one order of magnitude around a 1 µmol/l concentration in the tissue. This also ignores the problems of differential absorption of toxicant between lipid membranes and hydrophilic cytoplasm.

ent-Kaurene synthesis inhibition

EPTC and metolachlor are known to inhibit kaurene synthesis (Figure 8.2). EPTC inhibited GA production and kaurenoid synthesis (Wilkinson and Ashley, 1979). This reduction in GA synthesis was shown to be due to an

inhibition of GGPP cyclization to kaurene in a cell-free enzyme system (*Table 8.3*) (Wilkinson, 1982c). Concomitantly, R-25788 reversed the activity of EPTC in the inhibition of kaurene synthesis. Metolachlor has been used commercially in maize and soya bean for several years, but recently an antidote was introduced which safened the use of metolachlor in sorghum. When CGA-43089 is applied to sorghum seed 6 weeks prior to planting, metolachlor is not deleterious to sorghum (Gerber, Muller and Ebner, 1974). Without the safener, metolachlor is highly toxic to sorghum. This safening effect is at least partially due to a reversal, in seed treated with CGA-43089, of metolachlor inhibition of kaurene synthesis in sorghum coleoptiles (*Table 8.3*) (Wilkinson, 1981b).

Table 8.3 RELATIVE INCORPORATION OF ^{14}C -MEVALONIC ACID INTO *ent*-KAURENE IN AN ETIOLATED SORGHUM COLEOPTILE CELL-FREE ENZYME SYSTEM

Chemical	Concentration ($\mu\text{mol/l}$)			
	0	0.1	1	10
	% untreated			
EPTC	100	60	40	20
EPTC + R-25788	100	95	115	—
Metolachlor	100	10	10	20
Metolachlor + CGA-43089	100	300	300	300

Thus, both EPTC and metolachlor are shown to inhibit kaurene synthesis. Since both R-25788 and CGA-43089 induce an increased degradation of the respective herbicides, the activities of these antidotes require further research.

***ent*-Kaurene oxidation inhibition**

Ancymidol inhibits all of the reactions of kaurene oxidation (*Table 8.4*) (Coolbaugh, Hirano and West, 1978; Coolbaugh, Swanson and West, 1982; Wilkinson, 1982b). Ancymidol is used for dwarfing in the greenhouse flower industry as a post-emergence post-spray. A very close analogue (triarimol) is used as a fungicide, but does not induce any dwarfing of pea plants following post-emergence application (Coolbaugh, Swanson and West, 1982). Study of the activity of ancymidol, triarimol and several other analogues led to the conclusion that mediation of penetration of a compound into the plant as well as species differences in enzyme specificity were determinative factors in the establishment of activity in GA biosynthesis inhibition (Coolbaugh, Hirano and West, 1978). Thus, the chemicals might be active in cell-free systems but fail completely when sprayed on the plant. Concomitantly, activity may exist in one species and fail in another on the basis of differences in enzyme specificities for the same reaction in different plant species. This may be one more example of the selective herbicide activity.

Mefluidide is utilized to suppress growth, increase tillering in grasses, inhibit seedhead production and increase the sucrose content of sugarcane (Anon., 1979). In a cell-free enzyme system from sorghum coleoptiles, mefluidide produced a greater kaurene oxidation inhibition than did ancymidol (*Table 8.4*) (Wilkinson, 1982b). Since a decrease in GA content results in an

Table 8.4 RELATIVE INCORPORATION OF ^{14}C -MEVALONIC ACID INTO *ent*-16-KAURENOL IN A CELL-FREE ENZYME SYSTEM FROM ETIOLATED SORGHUM COLEOPTILES

Chemical	Concentration (mol/l)				
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}
		% untreated			
Ancymidol	100	—	70	40	35
Triarimol	100	—	—	100	50
Mefluidide	100	33	20	20	20
Alachlor	100	—	41	21	—
Alachlor + acetochlor	100	—	284	808	—

increase in peroxidase activity which degrades indole-3-acetic acid (IAA) and increases lignification, grasses which have an induced deficiency in GA demonstrate the following sequences in symptomology: (1) elongation ceases, (2) plants often turn dark green, (3) after a period of time, buds break through the side of the sheath (i.e. tillers) and (4) growth resumes. This chronology is explicable as: (1) deficiency of GA = growth cessation, (2) precursors inhibited from producing GA are utilized for chlorophyll, etc., production (*Figures 8.1* and *8.2*), (3) the antibiotic is degraded but the tissues which were originally treated have become lignified and cannot grow, so that buds break through the side of the sheath, and (4) growth resumes (usually as tillers). Dicotyledonous species follow the same symptom chronology, except that lateral leaf buds grow on the stem and, of course, the rate of antibiotic degradation is dependent upon the species involved.

Alachlor is a widely used herbicide in maize, soya bean, dry bean, potato, peanut and cotton. Recently, alachlor was shown to inhibit the oxidation of kaurenol and kaurenol was depleted in the sorghum coleoptile cell-free enzyme system (*Table 8.4*) (Wilkinson, 1982a). Concomitant application of acetochlor resulted in a restoration of growth and an accumulation of kaurenol (*Table 8.4*). Since kaurenol was extensively accumulated in the enzyme extract treated with alachlor + acetochlor, there are probably more sites of activity below kaurenol or within GA metabolism itself (*Figure 8.2*).

Tetacyclacis is a very recent addition to this group of kaurene oxidation inhibitors. Currently, it is being tested as a growth retardant in rice to be transplanted (Rieber and Jung, 1982). The metabolic degradation of this material requires 14 days and then the rice seedlings resume growth without the necessity of bud break of axillary buds. Thus, this compound seems to inhibit kaurene oxidation without the accompanying increase in peroxidase activity that occurs with other kaurenol syntheses and oxidation inhibitors. Clarification of this action could lead to other usable compounds.

Chemical structure relationships

Comparison of the chemical structures of EPTC, metolachlor, mefluidide, alachlor, ancymidol and tetacyclacis does not reveal discernible similarities that can be designated as the 'centre of activity' of these molecules. This is probably due to insufficient evidence as well as the general paucity of infor-

mation concerning the enzyme systems involved. Even if we had sufficient information concerning these enzyme systems in a single species, understanding the modes of activity in other species would require the same kind of data for each other species. Concurrently, degradation pathways, rates and species differences must be established.

Generally, it is accepted that the best 'antidotes' for any herbicide will closely resemble the structure of the active herbicide. Comparison of EPTC and R-25788, metolachlor and CGA-43089, or alachlor and acetochlor, tends to substantiate this hypothesis. Yet unpublished data from our laboratory indicates that compounds totally unrelated structurally will serve as antidotes when they influence the same enzymatic biosynthesis pathway at totally different sites of activity. Developments along this line of research will be very interesting and should be productive.

Prognosis

There are many chemicals currently being utilized as herbicides whose influence on plant growth, etc., lends credence to a hypothesis that they function as GA precursor biosynthesis inhibitors. Continued elucidation of the enzymatic activity, structure and specificity will greatly enhance our understanding of the mechanical and chemical processes involved in plant growth. Thus, this process termed 'plant growth' (i.e. enlargement) will, before too long, be understood with a precision equivalent to that currently attained with photosynthesis. In plant growth there remains much to be done, but an elementary introduction to a preliminary evaluation can now be written. GAs are a centre-piece of that introduction and GA biosynthesis inhibitors should help make the remainder attainable.

Acknowledgements

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FUNGAL ANTAGONISM IN RELATION TO PEACHES

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Introduction

Monilinia laxa (Aderh et Ruhl) Honey induces one of the main diseases of peach trees (Zehr, 1982). This fungus infects the aerial parts of host plants to give a variety of symptoms including blighting of blossoms, buds, leaves and twigs; cankers on woody tissues; and rotting of fruits (Heaton, 1972), although it is mainly a pathogen of blossoms and twigs (Byrde and Willetts, 1977).

Effective chemical control of this pathogen has not been achieved yet. The multicyclic nature of the disease it induces requires several applications of mainly site-specific fungicides each year. This then results in fungicide resistance problems (Tate *et al.*, 1974; Katan and Shabi, 1981; Gilpatrick, 1981; 1982).

Biological control offers the possibility of reducing such inconvenience by decreasing selective pressure on the pathogen populations. However, few attempts have been made in this direction (Byrde and Willetts, 1977). Since the development of biological control systems could be of importance within an integrated management control, antagonists against the pathogen *M. laxa* among the fungal microflora isolated from peach twigs and flowers have been screened.

Materials and methods

PLANT MATERIAL AND FUNGI

Twig cuttings of peach trees (*Prunus persica* L.) cv. 'Jeronimo', from the orchard of the E.T.S.I. Agrónomos in Madrid, were used. Throughout the experiments, the orchard did not receive any phytotherapeutic treatment.

The plant pathogen *M. laxa* (Aderh et Ruhl) Honey monosporic strain was isolated from infected apricot twigs from Almonacid de la Sierra (Zaragoza, Spain).

Fungi with potential antagonistic effect against *M. laxa* were isolated from twigs and flowers of peach trees grown in the E.T.S.I. Agrónomos orchard. The isolations were identified at genus level and some of them at species level,

by using traditional taxonomic methods. Those isolates which could not be included in any species were grouped in clusters on the basis of numerical taxonomy analysis (Diday, 1970; 1971; Sneath and Sokal, 1973). Within each species or cluster, one randomly chosen isolate was examined for *in vitro* antagonistic effects.

ESTIMATION OF ANTAGONISM *in vitro*

The antagonism between each of the 127 fungal species and clusters of isolates components of the microflora of peach twigs and flowers was tested *in vitro*.

Inhibition of *M. laxa* by fungi was evaluated in dual culture on 'Pronadisa' malt-extract agar (MEA) and 'Difco' potato dextrose agar (PDA). The percentage inhibition of radial growth and the width of the zone of inhibition were essentially determined, as described by Fokkema (1973) and Royse and Ries (1978). The mycelial plug of *M. laxa* was previously placed on the plates and incubated for 3 days before inoculating the potential antagonistic fungus. When colonies reached their maximum growth (13 days in MEA and 12 days in PDA) the zones of inhibition and the pathogen radial growth were measured. Four replicate cultures were included for each of the interactions studied. Data were statistically analysed by a Wilcoxon non-parametric range test.

To observe possible hyperparasitism and lysis phenomena, mycelial plugs from the contact zone between the pathogen and the potential antagonists were taken from dual-culture Petri dishes. These mycelial plugs, containing both the pathogen and the potential antagonist fungi, were transferred to fresh PDA media and incubated for 5 days at $20 \pm 1^\circ\text{C}$. When *M. laxa* did not grow, a second sample of mycelia from the contact zone of the dual-culture Petri dishes was observed under a light microscope.

EVALUATION OF ANTAGONISM *in vivo*

Fungal isolates resident on peach twigs and flowers which showed antagonistic properties against *M. laxa* in *in vitro* tests, were further tested in *in vivo* assays on peach twig cuttings under laboratory conditions.

The top 5 mm of cuttings were excised and the exposed surface was inoculated with a mycelial plug of *M. laxa* or the selected resident antagonistic fungi. The cuttings were introduced in test tubes provided with cotton wool plugs and containing 20 ml of sterile distilled water. After 4 days' incubation at $25 \pm 1^\circ\text{C}$, the mycelial plugs were replaced by a second plug of the antagonist or the pathogenic fungi and the test tubes were incubated for a further 20 days. Agar medium plugs were also inoculated as controls. The lesions produced in the cuttings were estimated by measuring the length of the damaged tissue. Six replicate cuttings were introduced in each treatment. After 8 and 24 days of total incubation time, the lesion sizes and the extent of pathogen invasion were determined. To determine the extent of invasion, the cuttings were surface sterilized and the top 25 mm were sliced into 5 mm thick pieces with a sterile scalpel. These pieces were placed on PDA media and the occurrence of growth was observed after 7 days of incubation at $25 \pm 1^\circ\text{C}$.

Results

In order to obtain fungi with antagonistic properties against the plant pathogen *M. laxa*, the epiphytic fungal microflora of peach twigs and flowers have been studied. Components of this microflora were isolated on agar media and 127 different species or clusters of isolates were identified by their morphological and physiological characteristics. Only 24 of these species or clusters included isolates that were significantly and consistently isolated and were designated resident fungi according to Leben (1965). *Penicillium*, *Aspergillus*, *Alternaria* and *Cladosporium* were the genera better represented among the resident flora.

One isolate from each fungal species or cluster was randomly chosen and

Table 9.1 MEAN PERCENTAGE INHIBITION OF RADIAL GROWTH OF *Monilinia laxa* BY EPIPHYTIC MICROFLORA FUNGI OF PEACH TWIGS AND FLOWERS IN DUAL CULTURE ON MALT-EXTRACT AGAR (MEA) AND POTATO-DEXTROSE AGAR (PDA)*

Fungi	Media	
	MEA	PDA
<i>Alternaria</i> X	0.00 $\pm$ 0.00	31.67 $\pm$ 0.68
<i>Alternaria</i> XV	34.00 $\pm$ 1.55	0.00 $\pm$ 0.00
<i>Alternaria</i> XVIII	7.50 $\pm$ 1.07	22.00 $\pm$ 1.51
<i>Alternaria</i> XX	6.75 $\pm$ 1.05	26.25 $\pm$ 0.87
<i>Penicillium</i> XXXIII	0.00 $\pm$ 0.00	26.00 $\pm$ 1.51
<i>Epicoccum purpurascens</i>	1.33 $\pm$ 0.57	29.50 $\pm$ 1.27
<i>Sordaria</i> sp.	10.50 $\pm$ 0.76	38.25 $\pm$ 1.31
<i>Botrytis cinerea</i>	4.50 $\pm$ 0.23	21.25 $\pm$ 0.87
<i>Cercosporidium chaetomium</i>	3.00 $\pm$ 0.87	28.50 $\pm$ 0.68
<i>Monilinia laxa</i>	0.00 $\pm$ 0.00	6.00 $\pm$ 0.57
Potato-dextrose agar	5.50 $\pm$ 0.98	3.75 $\pm$ 0.23

* Percentage inhibition of radial growth was determined, as indicated in the text, under Materials and Methods, and expressed as a mean of 4 replicate cultures $\pm$ standard deviation of the mean.

Table 9.2 ZONE OF INHIBITION (RANGE IN CM) PRODUCED BY EPIPHYTIC MICROFLORA OF PEACH TWIGS WITH *Monilinia laxa* ON MALT-EXTRACT AGAR (MEA) AND POTATO-DEXTROSE AGAR (PDA)

Fungi	Media	
	MEA	PDA
<i>Aspergillus flavus</i>	0	4-5
<i>A. sparsus</i>	0	4-5
<i>Penicillium</i> XX	0	1-2
<i>Penicillium</i> XII	4-5	4-5
<i>Penicillium</i> XV	1-2	5-6
<i>Penicillium</i> XXXII	0	4-5
<i>Penicillium</i> XXXVI	2-5	4-9
<i>Penicillium</i> XXXVIII	1-2	1-2
<i>Penicillium</i> XL	4-5	0
<i>Epicoccum purpurascens</i>	5-6	5-6
<i>Sordaria</i> sp.	4-5	5-6
<i>Cytospora</i> I	0	3-4
<i>Cytospora</i> II	4-5	4-5
<i>Cytospora</i> III	4-5	4-5
<i>Paecilomyces variotii</i>	0	2-3

tested *in vitro* for antagonistic effects against *M. laxa*. Nine out of 127 isolates tested exhibited inhibition of radial growth (Table 9.1) of *M. laxa* in dual culture on MEA and PDA media. With one exception, all isolates produced significant inhibition of radial growth on PDA and no significant effect was observed on MEA (Table 9.1). The exception is *Alternaria* XV which had no effect against *M. laxa* on PDA and exhibited a strong inhibition effect on MEA. Only two isolates (*E. purpurascens* and *Sordaria* sp.) showed antagonistic effects against *M. laxa* when tested by both methods: inhibition of radial growth and formation of inhibition zones. The medium has also a remarkable effect on the inhibition zones produced by most of the isolates (Table 9.2).

The tests carried out to demonstrate the potential hyperparasitism and lysis phenomena showed that *Aspergillus terreus*, *A. ficuum*, *A. nidulans*, *Penicillium* I, II and VII, *Alternaria* V and *Mucor racemosus* did not allow the pathogen growth and development. Three of them, *Penicillium* I, II and VII, produced lysis of the pathogen mycelium (Figure 9.1). Also, a thickness and granulation of the hyphal walls were observed.

The resident fungi showing antagonistic properties against the pathogen in *in vitro* assays were tested *in vivo*. These fungi were *Aspergillus flavus* (isolate No. 331), *Penicillium* XXXVIII (isolate No. 524), *Epicoccum purpurascens* (isolate No. 281) and *Sordaria* sp. (isolate No. 293) which produced zones of inhibition or inhibited the pathogen radial growth (Tables 9.1 and 9.2) and *Penicillium* I (isolate No. 909) and *Penicillium* II (isolate No. 828) which produced mycelium lysis.

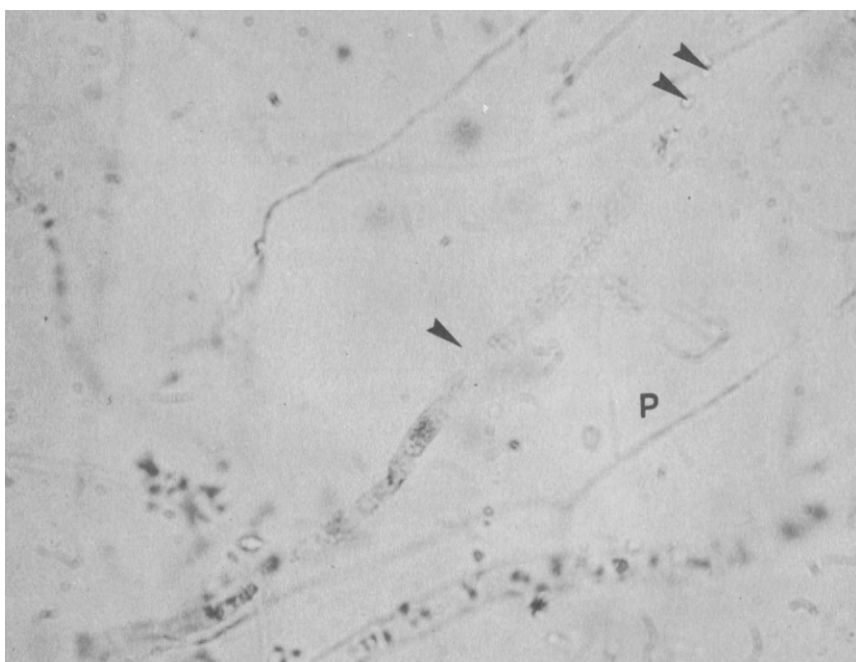


Figure 9.1 Light micrograph showing lysis of hyphae of *Monilinia laxa* (indicated by an arrow) in a dual culture with *Penicillium* I (indicated by a P)

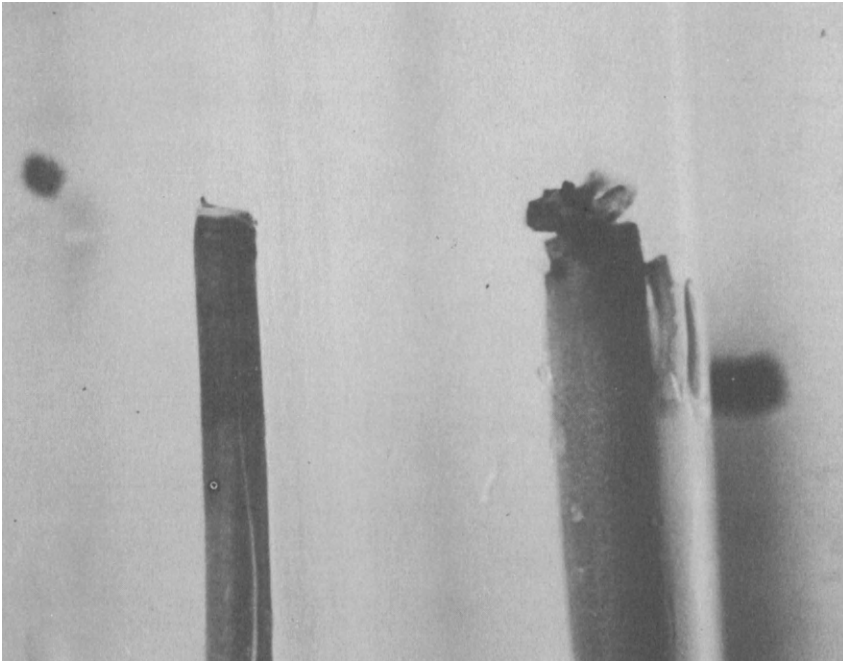


Figure 9.2 Peach twig cuttings showing lesions produced by inoculations of *Monilinia laxa* alone (left) and *M. laxa* and *Epicoccum purpurascens* (right)

Lesions produced in peach twigs were observed in all the inoculations with one or two fungi and with PDA (Tables 9.3–9.5). The lesions consisted of twig browning beginning in the inoculated surface (Figure 9.2). In some cases, exudate production could also be observed. Besides, lenticels were noticeable in some healthy areas of the twigs.

Table 9.3 shows the size of lesions produced in peach twig cuttings by

Table 9.3 SIZE OF LESIONS PRODUCED IN PEACH TWIG CUTTINGS BY INOCULATION OF *Monilinia laxa* OR FUNGAL ISOLATES*

Inocula†	Days after inoculation	
	8	24
<i>Monilinia laxa</i>	0.97 ± 0.06 a	3.67 ± 0.03 a
<i>Epicoccum purpurascens</i>	0.23 ± 0.04 b	0.47 ± 0.08 b
<i>Sordaria</i> sp.	0.57 ± 0.05 b	1.01 ± 0.09 b
<i>Aspergillus flavus</i>	0.51 ± 0.03 b	0.87 ± 0.02 b
<i>Penicillium</i> I	0.59 ± 0.08 b	0.93 ± 0.09 b
<i>Penicillium</i> II	0.61 ± 0.05 b	0.91 ± 0.02 b
<i>Penicillium</i> XXXVIII	0.88 ± 0.03 a	2.03 ± 0.11 a
Potato-dextrose agar	0.50 ± 0.09 b	0.83 ± 0.02 b

* The lesion sizes were estimated by the length (cm) of the damaged tissue. Values are means of 3 replicate cuttings ± standard deviation of the mean. Means followed by the same letter in a column are not significantly different ($P \geq 0.05$) by the Newman-Keuls range test.

† Cuttings were inoculated at 0 days and reinoculated at 4 days of incubation. The 6 fungal isolates examined are residents of peach twigs and flowers and have antagonistic properties against *Monilinia laxa* in *in vitro* assays.

Table 9.4 INVASION AND LESIONS PRODUCED BY *Monilinia laxa* IN PEACH TWIG CUTTINGS AS AFFECTED BY A SECOND INOCULATION OF FUNGAL ISOLATES

Inocula*	Days after inoculation			
	8		24	
	Lesion size† (cm)	Extent of pathogen invasion‡ (cm)	Lesion size† (cm)	Extent of pathogen invasion‡ (cm)
<i>Monilinia laxa</i> -Potato dextrose agar	0.90 ± 0.07 a	0.5	2.37 ± 0.05 a	1.5
<i>M. laxa</i> - <i>Epicoccum purpurascens</i>	0.90 ± 0.09 a	0.5	2.03 ± 0.12 a	1.0
<i>M. laxa</i> - <i>Sordaria</i> sp.	0.73 ± 0.03 b	1.0	3.27 ± 0.03 a	1.5
<i>M. laxa</i> - <i>Aspergillus flavus</i>	0.67 ± 0.06 b	0.5	2.00 ± 0.12 a	1.0
<i>M. laxa</i> - <i>Penicillium</i> I	0.73 ± 0.04 b	0.5	0.87 ± 0.01 b	n.r.
<i>M. laxa</i> - <i>Penicillium</i> II	0.87 ± 0.05 a	0.5	0.91 ± 0.07 b	n.r.
<i>M. laxa</i> - <i>Penicillium</i> XXXVIII	0.85 ± 0.01 a	1.0	2.89 ± 0.04 a	2.0

* Cuttings were inoculated at 0 days with *Monilinia laxa* and reinoculated with medium or fungal isolates after 4 days of incubation. The 6 fungal isolates examined are residents of peach twigs and flowers and have antagonistic properties in *in vitro* assays.

† See footnote (*) in Table 9.3.

‡ The extent of pathogen invasion was estimated, as indicated in the text, under Materials and Methods. Figures are means of values from 3 replicate cuttings; n.r. indicates no recovery of the pathogen.

Table 9.5 INVASION AND LESIONS PRODUCED BY *Monilinia laxa* IN PEACH TWIG CUTTINGS PREVIOUSLY INOCULATED WITH FUNGAL ISOLATES

Inocula*	Days after inoculation			
	8		24	
	Lesion size† (cm)	Extent of pathogen invasion‡ (cm)	Lesion size† (cm)	Extent of pathogen invasion‡ (cm)
<i>Monilinia laxa</i> -Potato dextrose agar	0.90 ± 0.07 a	0.5	3.30 ± 0.17 a	2.0
<i>M. laxa</i> - <i>Epicoccum purpurascens</i>	0.90 ± 0.09 a	0.5	0.73 ± 0.05 b	1.0
<i>M. laxa</i> - <i>Sordaria</i> sp.	0.73 ± 0.03 b	1.0	1.93 ± 0.09 a	2.0
<i>M. laxa</i> - <i>Aspergillus flavus</i>	0.67 ± 0.06 b	0.5	1.02 ± 0.09 b	0.5
<i>M. laxa</i> - <i>Penicillium</i> I	0.73 ± 0.04 b	0.5	0.95 ± 0.02 b	n.r.
<i>M. laxa</i> - <i>Penicillium</i> II	0.87 ± 0.05 a	0.5	0.61 ± 0.05 b	n.r.
<i>M. laxa</i> - <i>Penicillium</i> XXXVIII	0.85 ± 0.01 a	1.0	2.53 ± 0.06 a	1.5

* Cuttings were inoculated at 0 days with medium or fungal isolates and reinoculated with *Monilinia laxa* after 4 days of incubation. The 6 fungal isolates examined are residents of peach twigs and flowers and have antagonistic properties against *M. laxa* in *in vitro* assays.

† See footnote (*) in Table 9.3.

‡ See footnote (‡) in Table 9.4.

individual inoculations of *M. laxa* or the six resident antagonistic fungi after 8 and 24 days of incubation. All the antagonists, except *Penicillium* XXXVIII, produced significantly smaller lesions than the pathogen. These lesions were not significantly different from those produced by the PDA plugs.

Tables 9.4 and 9.5 illustrate the extent of pathogen invasion and the size of lesions produced in peach twig cuttings by *M. laxa* when the pathogen was

inoculated alone or in combination with the six antagonists tested. Two antagonists (*Penicillium* I and *Penicillium* II) significantly reduced the lesion sizes when inoculated after the pathogen, as compared with lesions produced by the pathogen alone (Table 9.4). Also, these two antagonists seem to eradicate *M. laxa* from the cuttings, since no recovery of pathogen was obtained after 24 days of incubation. No effect on lesion sizes was observed with the other four antagonists, although two of them (*A. flavus* and *E. purpurascens*) inhibited the extent of pathogen invasion (Table 9.4). When the antagonists were inoculated before the pathogen, four isolates (*Penicillium* I, *Penicillium* II, *A. flavus* and *E. purpurascens*) significantly reduced the lesion produced by *M. laxa* (Table 9.5). *Penicillium* I and *Penicillium* II were again capable of eradicating the pathogen from the cuttings. Also, *A. flavus* and *E. purpurascens* inhibited the extent of pathogen invasion. As in Table 9.4, *Sordaria* sp. and *Penicillium* XXXVIII had no effect either on lesion sizes or in the pathogen invasion capacity.

Discussion

The fungal microflora of peach twigs and flowers has been screened for antagonistic properties against the plant pathogen *M. laxa* by *in vitro* and *in vivo* assays.

The results of the tests *in vitro* demonstrate that 30 fungal components of peach twig and flower microflora affected the growth of the pathogen. Although the study of antagonism in culture plates presents obvious disadvantages (Baker and Cook, 1974), this methodology is still valid to obtain effective individual antagonists useful in an integrated biological control approach (Spurr, 1981).

Six out of the 30 fungal isolates showing antagonistic effects in *in vitro* assays were resident of peach twigs and flowers and were chosen for *in vivo* assays, since residents have been described of being capable of survival and growth in twigs for longer periods of time than the casual components of the fungal microflora (Blakeman and Fokkema, 1982).

Four of the six fungi (*Penicillium* I, *Penicillium* II, *A. flavus* and *E. purpurascens*) also interfere *in vivo* with the pathogenic capacity of *M. laxa*. When these fungi were inoculated alone they produced minimal lesions in twigs not significantly different from those produced by PDA plugs. This suggests that the lesions were due to the medium used in the inoculations and not to the antagonists themselves.

Penicillium I and *Penicillium* II prevented the formation of lesions in the twig cuttings when they were inoculated with *M. laxa* and the pathogen was not recovered from cuttings after 24 days of incubation (Tables 9.4 and 9.5). The eradication of the pathogen from the cuttings may be due to lysis of the mycelium by the antagonistic fungi, since disintegration of hyphal walls of *M. laxa* was observed in *in vitro* dual cultures. The lytic phenomena produced *in vitro* by both fungi was carried out without the direct contact between the hyphae of the pathogen and the antagonists. This fact suggests the formation of a lytic compound which disintegrates the pathogen cell walls. This substance should be diffused—to short distances—in the culture medium. Soil bacteria belonging to the genus *Arthrobacter* have been reported to produce

a similar kind of lysis on *Fusarium moniliforme* var. *subglutinans*, which induces cankers in pines (Barrows-Broadbent and Kerr, 1981). These bacteria also exert lytic effects against *F. roseum* by the action of a chitinase (Morrissey, Dugan and Koths, 1976) and against *Saccharomyces fragilis* by an extracellular lytic enzyme (Rowley and Bull, 1977). To our knowledge this is the first report about lysis of *Monilinia* by fungi, although Lockwood (1960) described lysis of mycelium of *M. fructicola* by natural soil.

Aspergillus flavus and *E. purpurascens*, on the other hand, seem to act through inhibition of *M. laxa* growth by the production of some fungistatic substance. They exhibited growth inhibition of the pathogen in *in vitro* assays (Tables 9.1 and 9.2), which points to antibiosis. *In vivo*, both fungi delayed the advancing of the pathogen through twig cuttings and decreased the lesion sizes (Tables 9.4 and 9.5). This effect is the more remarkable when the antagonistic fungi were inoculated before the pathogen, probably due to a higher accumulation of inhibitory substances in the twig cuttings. *Aspergillus flavus* and *E. purpurascens* are well known in the literature as producers of antifungal compounds. Slagg and Fellows (1947) found that *A. flavus* decreased severity of *Gaeumannomyces graminis* in natural infected soil. Also, Sanchez (1956) proved its antibiotic effect against *Phytophthora infestans*. Some of the antifungal compounds produced by *E. purpurascens* against certain plant pathogens have been identified as flavipin (Campbell, 1956; Bamford, Norris and Ward, 1961; Royse and Ries, 1978).

No antagonistic effect against *M. laxa* was observed *in vivo* with *Penicillium* XVIII and *Sordaria* sp. isolates, although these fungi inhibited the growth of the pathogen *in vitro*.

As a conclusion, the results presented here show that fungi components of the resident fungal microflora have the capacity of retarding or preventing the growth of *M. laxa* and may be useful in developing methods for biological control of this pathogen.

Note added in proof: *Penicillium* I and *Penicillium* II have been identified as *P. frequentans* and *P. purpurogenum* by Dr A.H.S. Onions (Commonwealth Mycological Institute, Kew, UK).

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AUREOFUNGIN IN PLANT DISEASES

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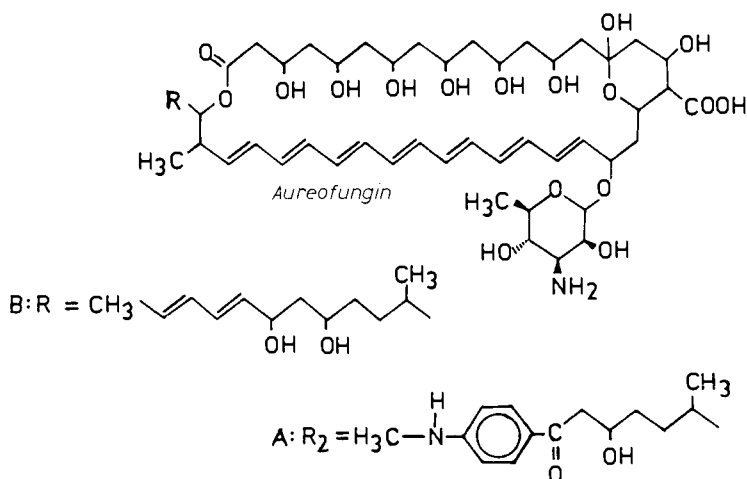
Most of the diseases of plants are caused by fungi and they outnumber other diseases. The fungi may be externally or internally seed-borne and soil-borne affecting leaves, shoots and finally grains and fruits in the field and, later, during storage. Fungicides and bactericides, either protectants or eradicants, are the main agents to protect the plants from diseases. Only recently have systemic fungicides, which are translocated upwards, come into prominence.

Since the discovery of penicillin by Fleming, antibiotics have been mainly used and screened for efficacy in human and animal diseases and only those which were of no therapeutic value were tried for the control of plant diseases. In addition, the toxicity of mercurial and other fungicides led scientists to look for antibiotics to control plant diseases. Antibiotics which are at present used for treating infections in human and animals are also being used in the agricultural field. However, a strong feeling exists that the antibiotics used for human therapy should not be used for controlling animal or plant diseases; therefore, regulatory committees are considering banning such use of those antibiotics. Thus, very few antibiotics will be available for the control of plant diseases. Japan uses the most antibiotics for controlling plant disease and, during 1975, the total amount of antibiotics used in Japanese agriculture cost \$17 million.

Aureofungin is an antifungal antibiotic discovered and developed at the Research Centre of Hindustan Antibiotics Ltd, Pimpri, Pune, India. It has been introduced into the Indian market exclusively for agricultural use. Earlier work on this antibiotic has been reviewed by Thirumalachar (1974).

Aureofungin belongs to the heptaene-polyene group (Thirumalachar *et al.*, 1964) and is produced by an actinomycete *Streptoverticillium cinnamomeum* var. *terricola* Thirum. The antibiotic is produced by submerged fermentation and isolated (Bhate and Acharya, 1964) from the mycelial cake using solvents. The compound has been well characterized and the structure shown on page 138 has been assigned (May Dean, 1976).

Aureofungin has either a *p*-amino acetophenone or *N*-methyl *p*-amino acetophenone moiety and is differentiated from others belonging to this group by the presence of mycosamine. It is a golden yellow powder, insoluble in water but soluble in alcohol, dimethylsulphoxide and dimethylformamide. It is readily soluble in dilute alkali.



Antimicrobial activity

The antimicrobial activity of Aureofungin can be tested *in vitro* by incorporating the antibiotic agar medium and streaking the test organism on it. In the case of *Sclerotia* in rust spores, they may be suspended in liquid nutrient medium containing different antibiotic concentrations and observed for germination. Solubilizing the antibiotic is very important. The definite quantity of the antibiotic is weighed and dissolved in 60% ethanol by adjusting the pH to 8.0–9.0 with dilute alkali, and then diluting serially with distilled water to get solutions of desired concentrations.

Although the antibiotic was previously dissolved in aqueous alcohol or dimethylformamide, before dilution with water for *in vivo* studies, now the antibiotic is marketed as Aureofungin-Sol which has to be dissolved in a small quantity of water and then diluted further for spraying as per requirement. Table 10.1 gives the *in vitro* minimum inhibitory concentration (MIC) of the antibiotic against various fungi.

Translocation

Any fungicide which is absorbed and translocated is always preferred for control of plant diseases. Aureofungin has the advantage over other chemicals in that it is absorbed and translocated in the plant body, both upwards and downwards. Methods for testing the systemic nature of fungicides are well laid down. Kadkol (1969) and Kadkol and Gopalkrishnan (1971) have demonstrated the translocation of Aureofungin. As Aureofungin has three sharp u.v. peaks, the part of the plant to be tested may be taken and the extracted juice examined under u.v. It has been observed that, as the antibiotic metabolizes, the peaks in the u.v. region disappear. Although polyenes are sensitive to light, when absorbed in the plant, biological activity is retained as chlorophyll prolongs the activity of polyenes.

Table 10.1

<i>Test organism</i>	<i>Minimum inhibitory concentration (µg/ml)</i>
<i>Alternaria brassicae</i>	1–2.5
<i>A. brunsii</i>	1
<i>A. citri</i>	5
<i>A. raphani</i>	2.5
<i>A. solani</i>	1–2
<i>A. tenuis</i>	0.5–1
<i>Aspergillus flavus</i>	2–10
<i>A. niger</i>	2–10
<i>Cercospora personata</i>	2
<i>C. carthami</i>	12
<i>Claviceps microencephala</i>	1–5
<i>Colletotrichum longisporum</i>	1–10
<i>Corticium saskii</i>	5
<i>Curvularia lunata</i>	0.5
<i>Dermatophora necatrix</i>	4
<i>Diaporthe citri</i>	1
<i>Fusarium moniliforme</i>	2.5
<i>F. oxysporum</i>	10
<i>Gibberella fujikuroi</i>	10
<i>G. zeae</i>	10
<i>Gleosporium lacticolor</i>	1
<i>Glomerella cingulata</i>	10
<i>Helminthosporium gramineum</i>	10
<i>H. nodulosum</i>	0.5–2
<i>H. maydis</i>	0.1–0.5
<i>H. oryzae</i>	0.1–0.25
<i>H. sigmoideum</i> var. <i>irregulae</i>	10
<i>H. turcicum</i>	0.5–1.0
<i>Macrophomina phaseoli</i>	2–5
<i>Phytophthora palmivora</i>	1–5
<i>P. citrophthora</i>	1
<i>Plenodomas lingam</i>	10
<i>Pyricularia oryzae</i>	0.05–1
<i>Pythium debaryanum</i>	1–5
<i>P. myrotilum</i>	5
<i>Protomyces macrosporus</i>	2.5
<i>Puccinia penniseti</i>	20
<i>P. recondita</i>	15
<i>Ozonium taxanum</i> var. <i>parasiticum</i>	1–5
<i>Ramularia cartlei</i>	10
<i>Rhizoctonia solani</i>	1–5
<i>Sclerotium rolfsii</i>	1–5
<i>Uromyces fabae</i>	15
<i>Ustilago tritici</i>	0.25
<i>Taphrina maculans</i>	2.5
<i>T. deformans</i>	0.5–1
<i>Verticillium alboatrum</i>	1–6

Toxicity

Toxicity of a fungicide, both to plants and operators spraying it, is very important. Most of the fungicides in use at present are known to be toxic to operators and some of them also cause burning of young shoots or reduce germination. In the case of Aureofungin, no adverse effects of any kind to plants or operators while spraying have been observed. Even at higher concentrations (1000–2000 p.p.m.), it does not affect the germination of seeds or growth of the plants.

In acute toxicity studies, 2 g/kg of body weight in mice and rats and 3.0 g/kg of body weight in rabbits and dogs orally were not toxic. Even chronic toxicity studies using 50 mg/kg for over 90 days showed no changes in organs or blood pictures.

Aureofungin in plant disease control

SEED-BORNE INFECTIONS

Seed-borne infections (which give poor germination), are numerous. The infective fungus might be externally or internally seed-borne. Externally seed-borne infection can be controlled by washing or seed dressing. But when the mycelium is internally borne beneath the seed coat, the treatment becomes very difficult, as on many occasions fungicides at active concentrations do not reach the mycelium. Aureofungin has given good protection and, in many cases, improved germination. The possibility of using Aureofungin in controlling a number of seed-borne infections was demonstrated by the work of Rahalkar and Neergaard (1969) at the Institute of Seed Pathology, Copenhagen, Denmark. They have classified the organisms by MICs (*Table 10.2*).

Table 10.2

MIC (µg/ml)	Organism
0.5	<i>Curvularia lunata</i> , <i>C. clavata</i> , <i>C. maculans</i> , <i>C. pallescens</i> , <i>C. protuberata</i>
1.0	<i>Alternaria brunsii</i> , <i>Aspergillus niger</i> , <i>Monilinia fructigena</i> , <i>Pyricularia oryzae</i>
2.5	<i>Fusarium equiseti</i> , <i>F. moniliforme</i> , <i>Drechslera halodes</i> , <i>D. maydis</i> , <i>D. oryzae</i> , <i>D. rostrata</i> , <i>D. sativa</i> , <i>D. tetramera</i> , <i>Nigrospora oryzae</i> , <i>Plenodomus lingam</i> , <i>Trichothecium roseum</i> , <i>Ullocladium lanuginosum</i>
5.0	<i>Colletotrichum</i> sp., <i>Fusarium semitectum</i>

They observed that the seeds infected by *Pyricularia oryzae*, when treated with 40 p.p.m. Aureofungin mixed with 20 p.p.m. CuSO₄, gave 57% germination but no infected seedlings. The germination percentage increased to 60% when the concentration of Aureofungin increased to 100 or 200 p.p.m. With untreated seeds which served as controls, the germination was 55–57%, but with 23% infected seedlings. As rice blast is a major disease, seed treatment to control seed-borne infection not only takes care of infection but also ensures better germination. Cabbage seeds infected with *Plenodomus lingam* on treatment with 100 p.p.m. of Aureofungin had considerably improved the germination.

Helminthosporium gramineum is an incitant of stripe disease of barley,

where mycelium is internally seed-borne. Dharam Vir and Raychaudhuri (1968) demonstrated that soaking the seeds in 25 p.p.m. Aureofungin solution for 2 h gave 100% control of the disease. In infected seeds, when plated out on nutrient medium, dark colonies of fungus emerged, whereas in treated seeds there was no fungal growth. In the field experiments, it was observed that, in the controls, there was 75% germination with 100% infection, whereas in Aureofungin-treated seeds germination was 95% with no infection at all.

Externally, seed-borne covered smut of oats is caused by *Ustilago hordei*. Dharam Vir and Raychaudhuri (1969) studied the effect of various fungicides in controlling smut of oats. Aureofungin treatment gave 100% control, while griseofulvin, actidione and mycostatin were effective to the extent of 60–80%. In the seeds treated with 20 p.p.m. of Aureofungin and mycostatin, the germination was above 90% with no infection, as against infected seeds which were not treated with any of these antibiotics, which gave 78% germination with 64% infection or actidione with 68% germination and 36% infection. Infected seeds which were not treated with any of these antibiotics gave 72% germination and 85% infected plants. Thirumalachar (1974) has shown that grain smut and head smut of sorghum are completely controlled by treatment with 100 p.p.m. Aureofungin for 30 min. The treated seeds can be planted immediately or stored for sowing later.

The large-scale cultivation of legumes often requires enrichment by nitrogen, and hence nitrogen-fixing bacteria are seed inoculated which helps in increasing nodulation. But, in many instances, the nodulation fails due to antagonistic action of soil fungi. Another observation is that some of the chemical fungicides used for seed dressing also inhibit such bacteria. Mukewar and Bhide (1969) have studied the action of fungicides and Aureofungin on rhizobia and have shown that seeds treated with 100 p.p.m. Aureofungin and inoculated gave a high percentage of nodulation. Similar results have been observed in the case of soya beans, peas, etc.

Helminthosporium oryzae is internally seed-borne and causes heavy seedling infection. Heavily infected seeds were treated with Aureofungin (Thirumalachar, 1967) and other fungicides. These results are summarized in Table 10.3.

Table 10.3

<i>Treatment</i>	<i>Germination (%)</i>	<i>Infected plants (%)</i>
Ceresan dry	68	13
Captan	60	20
Thiram	68	13
Aureofungin (20 p.p.m.)		
+ CuSO ₄	75	3
Aureofungin 100	80	2
Hot water	65	10
Control	60	80

They indicated a higher percentage of germination when 100 p.p.m. Aureofungin solution was used. Further studies have shown that it is possible to treat the seeds with 100 p.p.m. Aureofungin and store the grains for planting afterwards. This not only controlled infection of the seeds during germination, but retained their viability. Capoor and Marathe (1970) were able to

store citrus seeds for a long period—up to 200 days—using Aureofungin, while untreated seeds, which served as control, lost their viability rapidly due to infestation by saprophytic fungi during storage.

AUREOFUNGIN IN THE CONTROL OF DOWNY AND POWDERY MILDEW

Downy and powdery mildew are two important disease of various crops which reduce the vigour and yield of the crop. In the case of grapes downy mildew, caused by *Plasmopara viticola*, and powdery mildew, by *Uncinula necator*, along with anthracnose, by *Elsinoe viticola*, incur heavy losses. Bedi, Gurudip Singh and Suryanarayana (1969) have observed very good control of anthracnose by spraying 20 p.p.m. Aureofungin. Kadkol and Gopalkrishnan (1971) have demonstrated good control of downy mildew with an Aureofungin spray. Aureofungin in combination with CuSO_4 has also given excellent results in cases of powdery mildew.

Isabgul (*Plantago ovata*) is a medicinal plant and cultivated as a cash crop. The downy mildew *Perenospora plantaginis* is seed-borne and is so damaging that it sometimes becomes a limiting factor. Desai and Desai (1969) carried out large-scale trials in the field using Aureofungin. Seed treatment and spraying was observed to be an excellent treatment for controlling the infection and giving a profit of up to 225 rupees per hectare over the control. Gemawat and Prasad (1969) have used Aureofungin to control powdery mildew of cumin (*Erysiphe polygoni*) along with *Alternaria* blight. The results are summarized in Table 10.4.

Table 10.4

<i>Treatment</i>	<i>Blight control</i> (% control)	<i>Powdery mildew</i> (% control)	<i>Yield</i> (g/plant)
Cuman, 0.1% 1 spray	47	12	16.8
Cuman, 0.1% 2 sprays	53	31	26.3
Cuman + cosan 2 sprays	63	53	37
Aureofungin, 20 p.p.m. 2 sprays	56	62	36
Cosan, 0.2% 2 sprays	37	63	27.2
Karathane W.D., 0.2% 2 sprays	9	67	17
Dithane Z-78, 0.2% 2 sprays	45	47	27
Anteracol, 0.2% 2 sprays	42	18	25
Control (no fungicide)	0	0	8.0

It can be seen from Table 10.4 that Aureofungin alone could give control of both blight and powdery mildew, unlike cumen and cosan when used singly. In a recent study, increasing the Aureofungin concentration to 100 p.p.m., with CuSO_4 , has given 75–90% and 85–95% control of *Alternaria*

blight and powdery mildew, respectively. Many of the crops like cucurbits (gourds) are delicate and show injuries due to other fungicides. Aureofungin, at 50–100 p.p.m., with CuSO_4 , has given good results in controlling powdery mildew infections.

Piper betle leaf is used for chewing purposes and is an important cash crop. Based on laboratory results on the germination of conidia of powdery mildew (*Oidium piperis*), Jhamaria and Daftari (1970) have used Aureofungin to control the disease on the field scale and the results are summarized in Table 10.5.

Table 10.5

Treatment	Control (%)
Aureofungin, 25 p.p.m.	68
Elosol, 0.5%	58
Cosan, 0.2%	50
Thiovit, 0.5%	41
Water spray under pressure	18
Control (no spray)	0

Table 10.5 shows that Aureofungin gave the best results in controlling the powdery mildew. Venkat Rao and Narasimhan (1972) have used Aureofungin for treating betel leaves and packing material and observed good control of rotting during storage. The leaves stayed without blemish and attained a uniform yellow colour on the 9th day of storage. Agarwala and Thirumalachar (1967) have demonstrated the effectiveness of Aureofungin in controlling powdery mildew of apples caused by *Podosphaera leucotricha*. This was in comparison with other fungicides like cosan, solbar, thiovit, spitox, etc. In addition to powdery mildew, Aureofungin also controlled the other leaf spot diseases caused by *Cercospora*, *Pestalotia*, etc.

AUREOFUNGIN IN THE CONTROL OF FIELD CROPS AND OTHER CASH CROPS

Since Aureofungin is absorbed and translocated into the plant body both ways, there was a good possibility of using this in checking various diseases. Agarwala and Thirumalachar (1967) also found that Aureofungin can be used to control the root-rot of apple caused by *Dermatophora necatrix*, a soil invader. The disease is prevalent in Western countries also, where the perfect stage *Rossellini* is formed and the fungus is usually called *R. necatrix*. Sharma and Agarwala (1967) tried this on a field scale and showed that spraying the plant, drenching the collar and watering with 1.5 gal. of the same solution for each tree at the basin, allowed the plant to recover from the disease completely.

Aspergillus flavus—a common soil inhabitant—has assumed great importance due to its infestation of peanuts and excretion of aflatoxins B and G, which have carcinogenic properties. The peanut crop also suffers from tikka disease due to *Cercospora personata* and root-rot by *Sclerotium rolfsii*. Whitehead and Thirumalachar (1971) have shown that 40 p.p.m. Aureofungin, solubilized in soap and sodium lauryl sulphate, with 20 p.p.m. CuSO_4 , was

effective in controlling all the diseases. Two sprays were given—one at the time of flowering and another when the pods were formed. Aureofungin is readily absorbed from the leaves and is translocated to the roots. The antibiotic is secreted into the rhizosphere along with the root exudate, and the infestation of *A. flavus* is prevented.

Desai *et al.* (1966) carried out field trials using Aureofungin to control citrus gummosis incited by *Phytophthora palmivora* and *P. citrophthora*, which is world-wide in distribution. The plants were sprayed with 20 p.p.m. Aureofungin and the stem was swabbed with a 100 p.p.m. solution, both solubilized in liquid soap and sodium lauryl sulphate. Two to three treatments were given, depending upon the severity of the disease, at an interval of 15–20 days. The results were remarkable; not only did the gummosis disappear, but new healthy shoots came up bearing numerous flowers and fruits.

The early blight of potato, caused by *Alternaria*, is a severe disease affecting leaves and reducing the yield. Pawar and Sulaiman (1969) tested Aureofungin in the field in comparison with other fungicides. The results are summarized in Table 10.6.

Table 10.6

<i>Treatment</i>	<i>Concentration</i>	<i>Tuber yield</i> (kg/acre)
Captan	1 oz. in 2½ gal.	3134
Dithane Z-78	1 oz. in 2½ gal.	3230
Cuman	1 oz. in 2½ gal.	3077
Aureofungin	5 p.p.m.	3100
Control	—	2880

Thirumalachar (1968) showed that with 40 p.p.m. Aureofungin, in combination with 20 p.p.m. CuSO₄, the results were more encouraging. Late sprays also protected the tubers from getting *Macrophomina phaseoli* infection. Sulaiman, Lukade and Dawkhar (1966) have also shown the control of ergot of bajra due to *Claviceps microcephala*. Keshi and Mohanty (1968), while working with *Pyricularia oryzae* on *Elusine coracana*, compared 20 p.p.m. Aureofungin with other fungicides. Brestan-60 and Aureofungin had the best results, with increased yields of 24% and 28%, respectively.

Rusts and smuts cause enormous damage, especially to cereal crops. Hiremath and Pavgi (1971) studied the *in vitro* effect of Aureofungin on various species of rust genera, *Puccinia*, *Ravenelia*, *Uromyces*, etc. They found that the germination of spores was inhibited at 10–20 p.p.m. level. Mathur, Singh and Gupta (1971) have used Aureofungin to control the smut *Tolyposporium penicillariae* on pearl millet. Fungicides were sprayed 3 times at an interval of 10 days starting from the time of ear emergence. The results are summarized in Table 10.7.

Rao (1975) has demonstrated the efficacy of Aureofungin in controlling the wilt disease of coconuts incited by *Ganoderma lucidum*. This disease has become destructive in many coconut-growing areas. Drenching twice, at an interval of 15 days, the cut ends of roots, after exposing the root system to a depth of 20–30 cm with 1.5 g of Aureofungin-Sol in 5 l of water, gave good results.

Table 10.7

<i>Treatment</i>	<i>Yield (kg/ha)</i>
Zineb, 0.2%	3357
Ziram, 0.2%	3576
CuOCl, 0.3%	3180
Blitane, 0.3%	3538
Aureofungin, 3 g/acre	4511
Control	2500

In collaborative work at Georgia State University, the effect of Aureofungin on several root diseases inciting fungi was studied by Thirumalachar and Whitehead (1971). With Aureofungin, solubilized in soap and sodium lauryl sulphate, root-rot of ginger by *Pythium myrotilum*, *Rhizoctonia solani* infection of seedlings, *Macrophomina phaseoli* on cotton and *Ozonium texanum* var. *parasiticum* were effectively controlled. Sheath blight of rice caused by *Rhizoctonia solani* is potentially a serious disease. Kannaiyan and Prasad (1978) have used Aureofungin and compared the results obtained with other antibiotics. It was tested during two seasons (June to September and October to January). Two sprays were given at 0.1% level at a 10-day interval, i.e. 65 and 75 days of crop age. The results are given in Table 10.8.

Table 10.8

<i>Antibiotic</i>	<i>Grain yield (kg/ha)</i>	
	June–Sept.	Oct.–Jan.
Blasticidine EC	5000	4620
Kusumin	4830	4590
Aureofungin	5120	5440
Control	3370	3920

AUREOFUNGIN IN THE CONTROL OF POST-HARVEST DISEASES

Diseases of harvested crops during storage and transit are of great value, since the damage is considerable and direct. In India, where cold storage facilities are inadequate, the rotting during harvesting and transit is heavy.

Dharam Vir, Raychaudhuri and Thirumalachar (1967) have studied the *Diplodia* rot of mango. Since mango is an exportable item, the trade loses heavily. Dipping in 100 p.p.m. Aureofungin solution, of mangoes and paper strips, prolonged the life of mangoes without any blemishes. They also observed a similar effect in controlling *Alternaria* rot in tomato. Thirumalachar (1968) has shown effective control of *Oospora* rot of tomato and oranges with 80 p.p.m. Aureofungin dip treatment. Swarup and Raghava (1969) have shown that treating gladiolus bulbs or ginger bulbs with Aureofungin was the best method of preserving them against fungal rots during storage. Mention may be made of the successful control of brown-rot of peaches due to *Monilinia fructicola*. Whitehead and Chandler (1969) used Aureofungin as pre-harvest spray and post-harvest dip treatment. At 400 p.p.m., Aureofungin was comparable with captan but without any discoloration or blemishes.

Bananas are also exported from India. Aureofungin dip has given good protection against banana rot due to *Fusarium moniliforme* and *F. roseum*. Agarwal, Khara and Thind (1982) got 100% control of mandarin fruit rot by *Botryodiplodia theobromae* by using 0.5% Aureofungin as pre- and post-inoculation treatment. Sinha, Jayrajan and Kapoor (1972) observed that, compared to Bordeaux mixture, Cupramar, Dithane Z-78 and Ferban, Aureofungin sprays gave very good control of pathological pre-harvest fruit drop in sweet oranges.

Other investigations which have been completed using Aureofungin include control of apple scab (*Venturia inaequalis*); peach stem canker (*Valsa leucotricha*); peach leaf curl (*Taphrina deformans*); cherry leaf spot (*Coccomyces hiemalis*); and apple root-rot (*Xylaria malorum*).

Aureofungin has thus filled the gap in the need for a safer and systemic antibiotic for plant disease control from seed to post-harvest storage. A number of plant pathologists have contributed to the usefulness of Aureofungin and Hindustan Antibiotics owes them a debt of gratitude.

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THE EFFECT OF WOUND DEPTH ON THE POST-HARVEST *PENICILLIUM* STORAGE ROTS OF CITRUS

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Introduction

Citrus fruits are the principal agricultural export crop of Israel, with an annual sales value of approximately \$250 million. As in other citrus-producing areas, the Israeli citrus fruits are subject to very serious post-harvest fungal rots (Dawson and Eckert, 1977; Gutter, 1977; Pelsner, 1977; Smoot, 1977) and the problem of reducing the percentage of citrus fungal rots in the Israeli storage and export marketing distribution channels is, therefore, of major economic importance.

Israeli citrus are exported mainly to Western Europe, with smaller quantities to Eastern Europe and the Far East. These exports are processed in some 70 Israeli packinghouses, where the fruit undergoes, among other operations, a disinfection and chemical fungicide-wax treatment with the aim of controlling the citrus rot fungi. These packinghouse treatments are of cardinal importance to the final condition of the citrus fruits in the export market, and in many cases are the difference between success and failure of the exported fruit.

Moulds are the principal cause of citrus rots, principally *Penicillium digitatum* Sacc. (green mould), *P. italicum* Wehmer (blue mould), *Geotrichum candidum* Lk. ex. Pers. (sour rot), *Alternaria citri* Ell. and Pierce, *Diplodia natalensis* Pole-Evans (stem-end rot) and *Phytophthora citrophthora* (Sm. and Sm.) Leonian (brown rot) (Eckert, 1978a).

The present procedures for the control of these moulds involve the use of sodium *o*-phenylphenate (SOPP), thiabendazole (TBZ) and, to a lesser extent, benomyl, 2-aminobutane (2-AB), biphenyl and imazalil (Eckert, 1978a). These chemical fungicides are used in the initial packinghouse disinfection treatment and/or in the final wax treatment in concentrations and conditions depending on the fruit cultivar, degree of maturity, time of the growing season and designated export country.

The chemicals most commonly used in these treatments are SOPP and TBZ. SOPP is an effective fungicide; however, it is phytotoxic and its use is limited to the initial disinfection step. TBZ is also an effective fungicide and has become the principal protection against the decay fungi, especially *P. digitatum* and *P. italicum*, during storage, transportation and marketing. It is

usually used in the final wax treatment of the fruit. Sometimes, however, it is also used in the initial drencher step. TBZ has been approved by many countries for the post-harvest treatment of citrus fruits and, accordingly, has become the most widely used systemic benzimidazole fungicide for this purpose.

Recently, however, there have been disturbing indications of the development of increased resistance by citrus rot fungal strains, principally *P. digitatum* and *P. italicum*, to these synthetic chemicals, especially to TBZ. As a result, there has been an increase in the percentage decay in the stored and shipped fruit. In addition, there have been indications of increased decay caused by *Geotrichum candidum* and *Alternaria citri*—citrus pathogens which are not susceptible to TBZ at all. Prior to the use of TBZ and other synthetic benzimidazole fungicides, these two decay fungi caused only minor fruit losses. Now, with the extensive use of TBZ, these two fungi have become more prevalent, causing increased losses of fruit. This situation of resistance and cross-resistance to the benzimidazole fungicides, and the shift in fungal flora, has become a source of considerable concern in all citrus-growing areas (Smoot and Brown, 1974; Kuramoto, 1976; Houck, 1977; McDonald, Risse and Hillebrand, 1979).

The number of available substitute synthetic chemical fungicides for the control of citrus post-harvest rots is limited, as most products are not sufficiently effective, or approved for use. As a result, the present situation calls for the urgent need to develop new products and alternative approaches to these serious fungal rots, with particular cognizance of the threat of resistance.

We have been working on the development of biotechnological approaches for the control of these citrus fungal storage rots, and in our studies have been able to demonstrate the importance of wound depth relative to handling and treatment conditions.

Materials and methods

CITRUS FRUIT

Untreated Navel, Shamouti and Valencia oranges were received at different times during the citrus-growing season from the regional packinghouses of the Israel Citrus Marketing Board. The oranges were washed, without detergent, dried and stored at 4–5°C until used.

CULTURES

Strains of *Penicillium digitatum* Sacc. and *P. italicum* Wehmer were used in this study. The thiabendazole-sensitive strains of *P. digitatum* (coded 26-21-68) and *P. italicum* (coded 26-21-81) were isolated from infected oranges. The thiabendazole-resistant strains of *P. digitatum* (coded 26-21-61) and *P. italicum* (coded 26-21-73) were received from A. Shachnai of the Israel Citrus Marketing Board. All cultures were maintained on potato-dextrose agar, transferred periodically and just prior to use. The subcultures were incubated at 20–23°C for 7 days.

Wheat bran spore cultures of the *Penicillium* strains were prepared (per

250 ml Erlenmeyer: wheat bran 5 g, water 6.5 ml; no pH adjustment; sterilized 45 min at 121°C) by inoculation from a sporulated agar slant culture and were incubated 7 days at 20–23°C. The cultures grew uniformly throughout the bran and sporulated very well. Spore suspensions were prepared by shaking the individual sporulated ban cultures with sterile dilute agar solution (0.125% (w/v); 90 ml) and pipetting off the suspended mould spores. A sterile stainless mesh sleeve (1 mm sq. openings) fitted on the end of the pipette prevented the inclusion of any bran particles. Spore counts were determined using a Petroff-Hauser cell counter and were approximately 100 million spores per ml for *P. digitatum* and 200 million spores per ml for *P. italicum*. The spore suspensions were diluted, before use, to the indicated concentrations with 0.125% (w/v) agar solution.

FRUIT TREATMENTS

A polyurethane fruit separator form was positioned in the bottom of a heavy-duty cardboard box (40 cm length, 30 cm width and 12 cm height, prepared from the bottom part of a citrus shipping carton) and the oranges arranged in it in 3 columns of 4 rows, not touching each other or the box walls (*Figure 11.1*). A line was then drawn with a black felt pen, from the stem end half-way down each fruit, dividing the fruit visually into two portions.

The fruits were inoculated, as described in the individual experiments, by dipping a cork, containing protruding needles, into the inoculum suspensions (10 ml; spore concentration as indicated) and wounding the individual fruits at 6 separate sites, 3 on each side of the dividing line. The inoculating units were prepared with three 0.7 mm dia. needles, spaced 0.5 cm apart in a triangle, with the protruding length as indicated in the individual experiments.

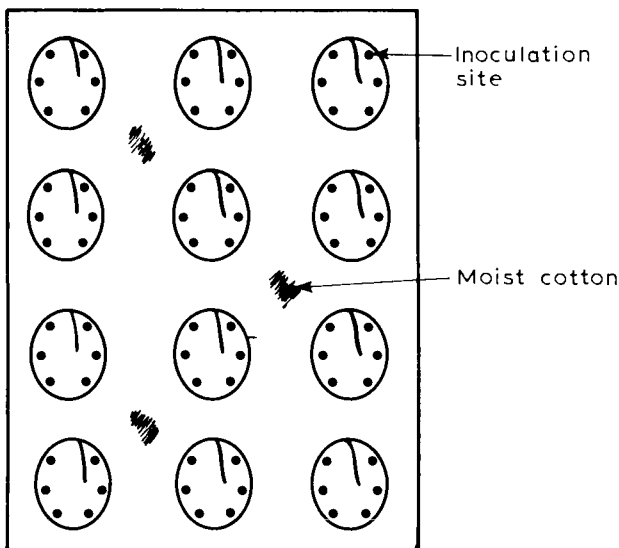


Figure 11.1 Arrangement of oranges in fruit treatment box

The inoculation sites were approximately 4 cm away from the dividing line and located close to the stem end, the equatorial diameter and the bottom end.

The inoculations were carried out by pressing the inoculating cork firmly into the fruit peel, twisting it through a 45° angle, and then pulling it out, to be dipped again in the spore suspension before inoculating the next site. To avoid the possible carry-over of chemical constituents from the fruit flavedo (exocarp) area, the inoculum suspension was replaced after every third inoculated fruit.

Moist cotton wads were then placed in 3 locations in each box, to maintain high humidity, and the entire box sealed in a clear plastic polyethylene bag. Since only the bottom part of the box was used, the inoculated fruits were clearly visible through the clear plastic. The boxes, containing the inoculated fruit, were then placed in a constant temperature room at 19–21°C for the indicated periods.

The fruits were examined daily and the first sign of infection, depending on the experimental conditions, was usually noticeable by the third day. Growth measurements were made using a centimetre ruler and the averaged data for the six individual inoculation sites expressed as a percentage of visible infection of the entire orange. The moist cotton wads were removed once the infections were well established.

FUNGICIDES

The following fungicides were evaluated: thiabendazole (TBZ) 2-(4'-thiazolyl)benzimidazole, 98% technical, Merck & Co; sodium *o*-phenylphenate (SOPP), Dow Chemical Co.

Results

EFFECT OF WOUND DEPTH

The effect of wound depth on the incidence and spread of *Penicillium* infections in oranges was investigated experimentally using Navel, Shamouti and Valencia orange varieties inoculated with TBZ-sensitive and TBZ-resistant strains of *P. digitatum* and *P. italicum* at controlled wound depths of 1.0, 1.5 and 2.0 mm.

The data indicated that oranges wounded at these depths, using the standard 10 million spores per ml inoculum level, always succumbed to the fungal infection. The 1.0 mm depth, however, invariably showed a delayed infection response, as compared to the spread of infection at 1.5 and 2.0 mm wound depths. This is readily seen in *Figure 11.2* for Shamouti oranges inoculated with *P. digitatum*. The spread of *P. digitatum* infection under these experimental conditions is always extremely rapid and is usually completed in 6–7 days.

The spread of *P. italicum* infection, on the other hand, is usually considerably slower, as can be seen by the results for Shamouti (*Figure 11.3*) and Valencia (*Figure 11.4*) oranges.

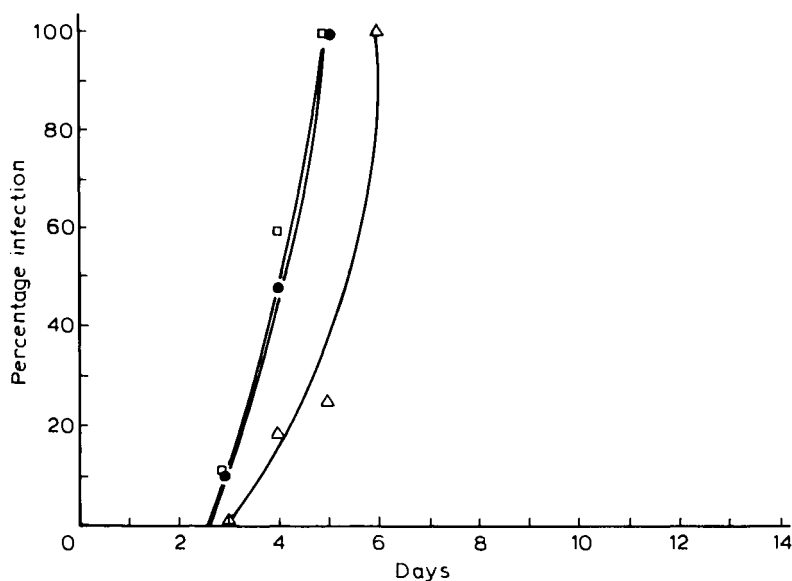


Figure 11.2 The effect of wound depth using Shamouti oranges wounded and inoculated with *Penicillium digitatum* TBZ-sensitive strain, 10×10^6 spores per ml; 1.0 mm (△), 1.5 mm (□) and 2.0 mm (●) wound depth

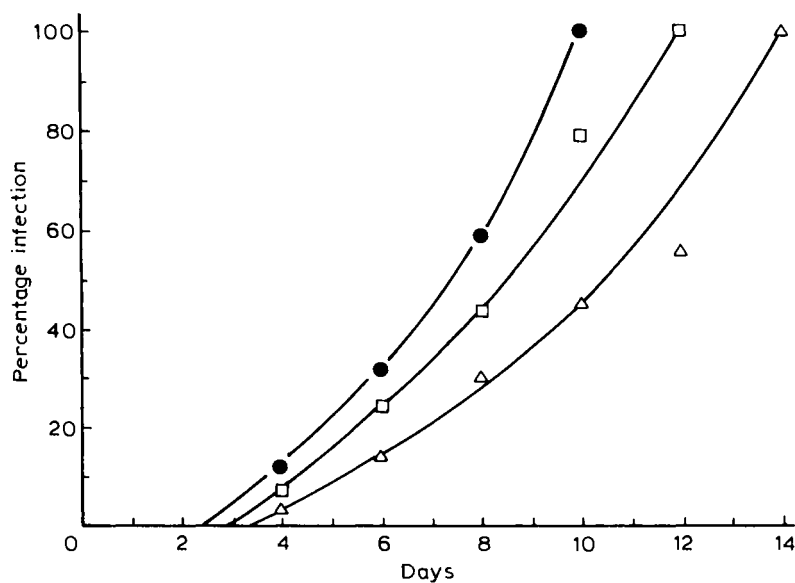


Figure 11.3 The effect of wound depth using Shamouti oranges wounded and inoculated with *Penicillium italicum* TBZ-sensitive strain, 10×10^6 spores per ml; 1.0 mm (△), 1.5 mm (□) and 2.0 mm (●) wound depth

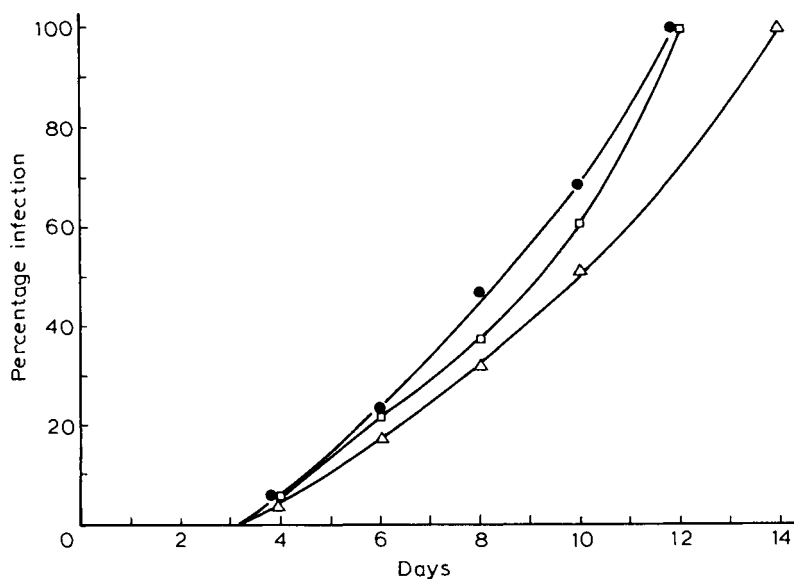


Figure 11.4 The effect of wound depth using Valencia oranges wounded and inoculated with *Penicillium italicum* TBZ-sensitive strain, 10×10^6 spores per ml; 1.0 mm (△), 1.5 mm (□) and 2.0 mm (●) wound depth

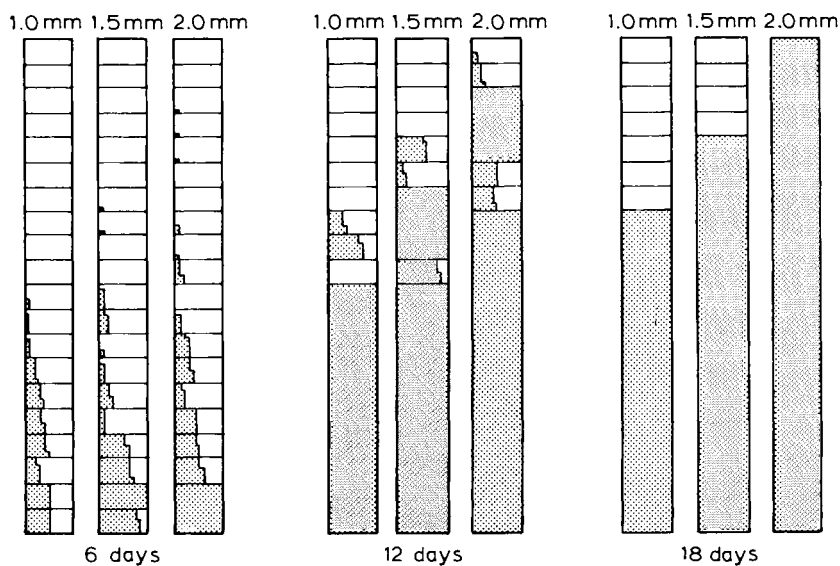


Figure 11.5 The effect of wound depth and inoculum density using Valencia oranges wounded and inoculated with *Penicillium digitatum* TBZ-sensitive strain; inoculum suspension diluted 1-2 in 20 dilution steps, from 10×10^6 to 19 spores per ml; 1.0 mm, 1.5 mm and 2.0 mm wound depths

EFFECT OF INOCULUM DENSITY

The effect of inoculum density on wound depth was investigated in a series of experiments and was found to mediate the infection rate and degree of infection. This is shown by the data in *Figure 11.5* for Valencia oranges inoculated with a *P. digitatum* inoculum dilution series, starting at 10 million spores per ml and decreasing in 1:2 dilution steps, to a final concentration of 19 spores per ml. As indicated, the fungal infection progressively develops in the inoculated series, more rapidly in the 1.5 and 2.0 inoculation depths, and is a function of depth, inoculum concentration and time. At the end of 18 days, all the 2.0 mm depth inoculated Valencia oranges were infected down to the 19 spores per ml level. In contrast, the 1.5 mm depth was free of infection below 305 spores per ml and the 1.0 ml depth below 2440 spores per ml.

P. digitatum TBZ-sensitive and TBZ-resistant strains were compared in

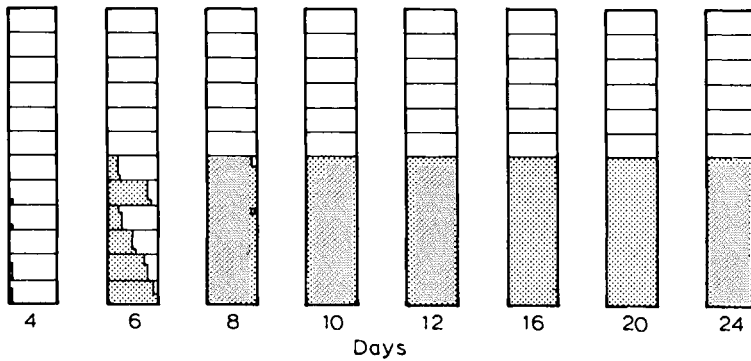


Figure 11.6 The effect of inoculum density using Shamouti oranges wounded and inoculated with *Penicillium digitatum* TBZ-sensitive strain; inoculum suspension diluted 1-3 in 12 dilution steps, from 10×10^6 to 56 spores per ml; 1.0 mm wound depth

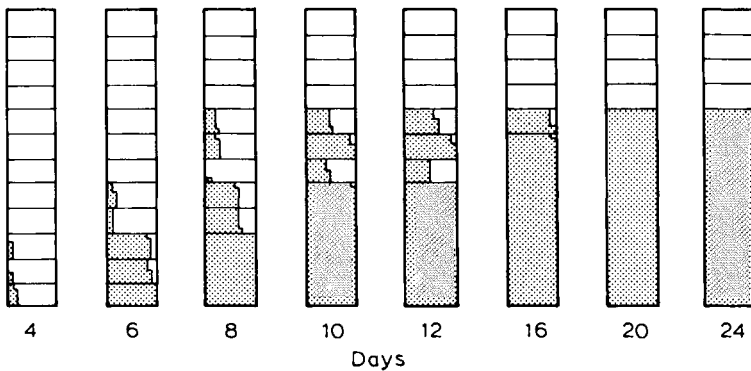


Figure 11.7 The effect of inoculum density using Shamouti oranges wounded and inoculated with *Penicillium digitatum* TBZ-resistant strain; inoculum suspension diluted 1-3 in 12 dilution steps, from 10×10^6 to 56 spores per ml; 1.0 mm wound depth

similar experiments using Shamouti oranges. In these experiments, the oranges were only wounded at the 1.0 mm depth with inoculum solutions prepared in a 1:3 dilution series, from 10 million spores per ml to 56 spores per ml. As indicated by the data in *Figures 11.6 and 11.7*, the infection picture, while similar to the results with Valencia oranges, shows some important differences; the TBZ-sensitive strain only infected the oranges down to 41 150 spores per ml, in contrast to the TBZ-resistant strain which infected the oranges down to the 4570 spores per ml level.

WOUND TISSUE RECOVERY

The effect of wound tissue recovery was investigated in a series of experiments using the *P. digitatum* and *P. italicum* TBZ-sensitive strains and Valencia oranges. In these experiments, the oranges were wounded with sterile needle assemblies at 1.0, 1.5 and 2.0 mm depths in the usual manner, but without the *Penicillium* spore solutions, which were then applied afterwards to the wound sites of individual oranges at 0, 1, 2, 4, 6 and 8 days. Control oranges were also inoculated at 0 days with needles dipped in the spore solutions in the standard manner. As shown by the data in *Figure 11.8* for the *P. digitatum* inoculated series, the positive effect of wound tissue recovery, mediated by the influence of inoculation depth, was clearly evident; the oranges wounded at the 2 mm depth eventually were all infected, while the oranges wounded at 1.5 mm and 1.0 mm depths showed delayed or no infection after 0–1 days.

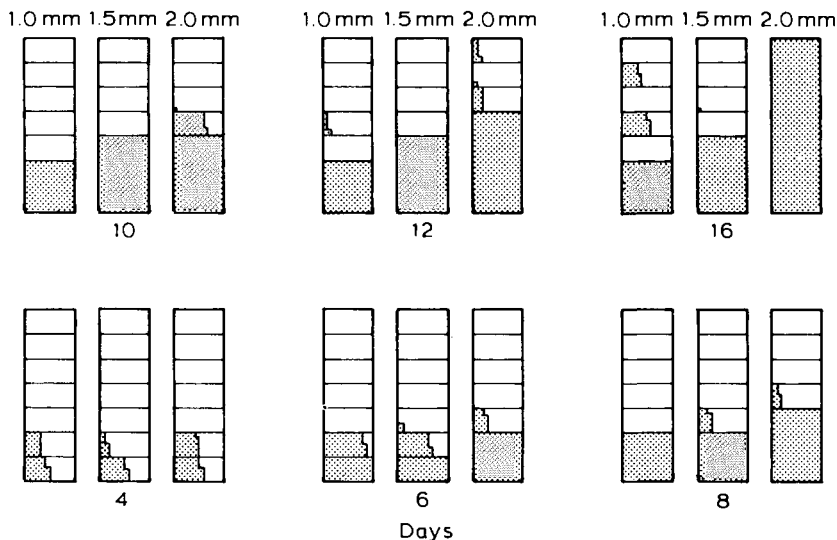


Figure 11.8 The effect of wound tissue recovery using Valencia oranges wounded at 1.0 mm, 1.5 mm and 2.0 mm depths and inoculated with *Penicillium digitatum* TBZ-sensitive strain, 10×10^6 spores per ml; control wounded with spore suspension at 0 days; other oranges wounded at 0 days and then individually treated with the spore suspension after 0, 1, 2, 4, 6 and 8 days

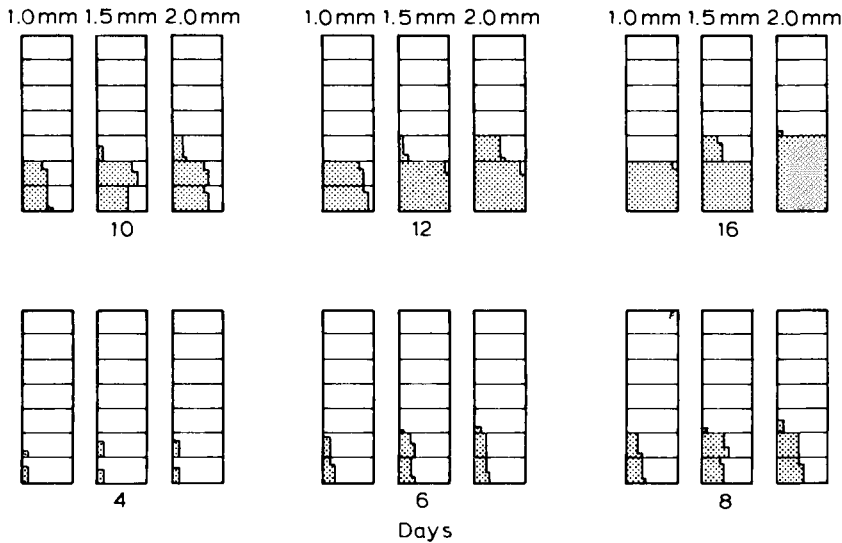


Figure 11.9 The effect of wound tissue recovery using Valencia oranges wounded at 1.0 mm, 1.5 mm and 2.0 mm depths and inoculated with *Penicillium italicum* TBZ-sensitive strain, 10×10^6 spores per ml; control wounded with spore suspension at 0 days; other oranges wounded at 0 days and then individually treated with the spore suspension after 0, 1, 2, 4, 6 and 8 days

The results with *P. italicum* (Figure 11.9) were even more dramatic; the 1.5 and 2.0 mm wound depth oranges showed no infection after a 1-day delay, while the 1.0 mm showed no infection after 0 days.

FUNGICIDE TREATMENTS

The use of the standard fungicides for the post-harvest treatment of oranges was then investigated. In the first experiment, sodium *o*-phenylphenate (SOPP) was used in a delayed treatment study, where Valencia oranges were inoculated with *P. digitatum* TBZ-resistant at 0 days, and individual oranges then treated with 0.5% SOPP after 0, 1 and 2 days. As indicated by the data in Figure 11.10, SOPP, under the most optimum of conditions, only delayed the infection but did not prevent the spread of the fungus to all treated oranges, regardless of wound depth.

In another series of experiments, the use of TBZ was investigated using *P. digitatum* and *P. italicum* TBZ-resistant cultures. The data was quite dramatic; despite the use of 2000 and 4000 p.p.m. TBZ, all oranges inoculated with *P. digitatum* became infected regardless of inoculation depth (Figure 11.11). With *P. italicum*, only 1 treatment (1.0 mm depth; 4000 p.p.m. TBZ) remained free of infection after 24 days; all the other treated oranges became infected (Figure 11.12).

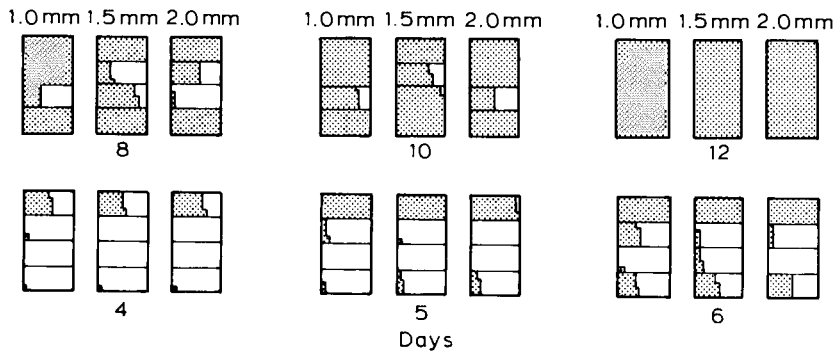


Figure 11.10 The effect of SOPP treatment of Valencia oranges wounded and inoculated with *Penicillium digitatum* TBZ-resistant strain, 10×10^6 spores per ml; 1.0 mm, 1.5 mm and 2.0 mm wound depths; all oranges wounded with spore suspension at 0 days and then individually treated with SOPP solution, followed by a water rinse, after 0, 1 and 2 days

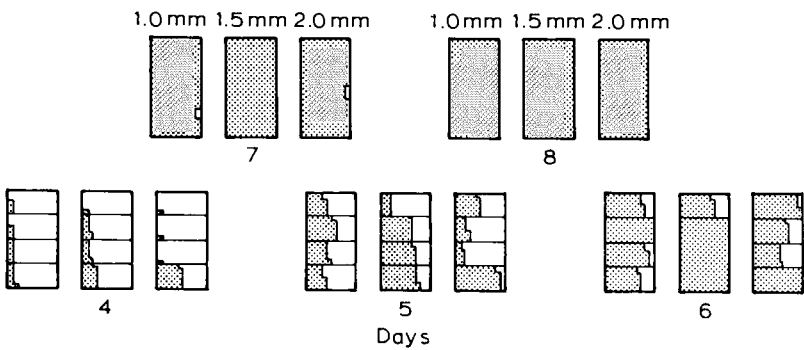


Figure 11.11 The effect of TBZ treatment of Navel oranges wounded and inoculated with *Penicillium digitatum* TBZ-resistant strain, 10×10^6 spores per ml; 1.0 mm, 1.5 mm and 2.0 mm wound depths; oranges treated 24 h later individually with water, propylene glycol, 0.2% TBZ in propylene glycol and 0.4% TBZ in propylene glycol

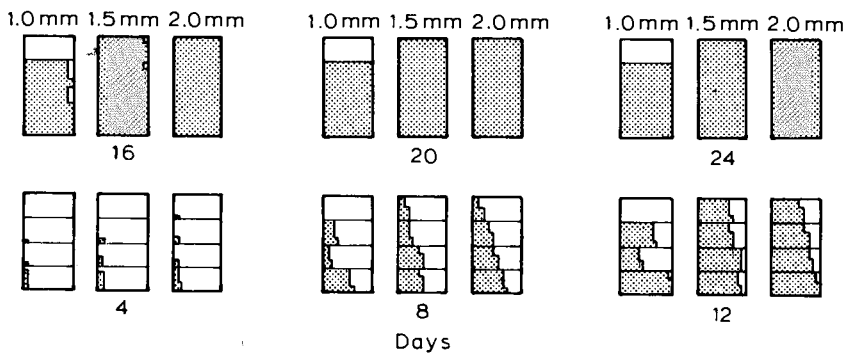


Figure 11.12 The effect of TBZ treatment of Navel oranges wounded and inoculated with *Penicillium italicum* TBZ-resistant strain, 10×10^6 spores per ml; 1.0 mm, 1.5 mm and 2.0 mm wound depths; oranges treated 24 h later individually with water, propylene glycol, 0.2% TBZ in propylene glycol and 0.4% TBZ in propylene glycol

Discussion

Citrus, a non-climacteric fruit type, has a relatively long post-harvest storage life (Schiffmann-Nadel, 1977) and invariably succumbs to fungal infections before physiological breakdown would make them unmarketable (Grierson and Hatton, 1977).

The fungal infections which develop during the post-harvest period are usually related to mechanical and physiological injuries which occur during harvesting, processing and packaging. A certain amount of injury is inevitable even with the most stringent care, and avoiding these injuries and subsequent losses is dependent on an understanding of the infection process, the host-pathogen relationship and the success of fungicide control treatments.

Our studies indicate that the severity of infection in the post-harvest situation is primarily a function of the pathogen, inoculum density, wound depth and fruit cultivar.

The effect of wound depth, while of apparent obvious importance, has not been fully investigated in the past and, as a consequence, various methods have been used to experimentally infect oranges with *P. digitatum* and *P. italicum* and to simulate processing injuries. These methods have included the use of a rotating circular saw making an approximate 1 mm deep wound (Roistacher and Klotz, 1955; Eckert, Kolbezen and Kraght, 1969), nail punctures 3 mm deep (Morris, 1982), injection by microsyringe 3 mm deep (Wild and Eckert, 1982), and injection into the pulp area (Eckert and Kolbezen, 1977). Other methods, such as scalpel cuts 4 mm deep (Barmore and Brown, 1979), and the excising of a section of the flavedo prior to inoculating the albedo, have also been used (Gutter *et al.*, 1981).

Our data have clearly shown the critical nature of wound depth, even with *P. digitatum* infections, which develop at an extremely rapid rate and tend to blur experimental conditions. The effect of wound depth for *P. digitatum* and *P. italicum* infections has been observed for Navel, Shamouti and Valencia oranges harvested at different times during the growing season, as well as for Michal mandarins, and a similar relationship has been found for *Geotrichum candidum* (J. Ziffer, unpublished).

The effect of wound depth is readily demonstrated using the standard inoculum level of 10 million spores per ml (Figures 11.2–11.4). However, the effect is even more dramatic when lower inoculum levels are used (Figures 11.5–11.7). While wound depth is still the critical factor, the concurrent reduction in inoculum level results in an apparent freedom from infection for long storage periods. These data undoubtedly reflect the physiological resistance of the fruit to low infection stress and suggest the possibility of enhancing the fruit's natural defences by improving processing conditions. This becomes an important consideration in projecting alternative protection procedures.

The ability to vary wound depth concurrently with inoculum density has added significance with regard to demonstrating differences in pathogen virulence and cultivar resistance to infection. The difference in infection rates observed for Shamouti oranges as compared to that for Valencia, and also the differences between *P. digitatum* TBZ-sensitive and TBZ-resistant strains, are significant (Figures 11.5–11.7). A similar situation has been found for Navel oranges which showed a high degree of resistance to infection at the

1.0 mm wound depth (J. Ziffer, unpublished). Navels are an early ripening fruit and this apparent resistance to infection could be a reflection of strong homeostatic responses before the onset of senescence.

The data for apparent wound recovery (*Figures 11.8 and 11.9*) are equally significant as these oranges remained uninfected even though they were in close proximity to heavily infected fruit and were contaminated on their outer surfaces with a light coating of spores (as demonstrated with cotton swabs). These results for apparent wound tissue recovery could be conditioned by phytoalexin synthesis, which also could be the factor mediating the effect of wound depth. Similar citrus results were recently reported in Florida, suggesting a lignification defence mechanism (Brown, Ismail and Barmore, 1978).

The fungicides SOPP and TBZ continue to be used in citrus packinghouses despite continued problems related to storage rot protection (McDonald, Risse and Hillebrand, 1979; Gutter *et al.*, 1981). Our data for SOPP indicated that the oranges treated with the fungicide 2 days after inoculation actually infected at a more rapid rate than did the water control, and that this chemical was not very effective. Since oranges usually are not processed immediately after picking, the use of SOPP could be less effective than is believed.

While SOPP is still considered to be an effective fungicide, unexplained time-treatment differences for *P. digitatum* (Eckert, 1969; Eckert, 1978b) and *G. candidum* (Wild, Rippon and Nicholls, 1976) suggest that its use should be re-evaluated.

The problem with TBZ, as illustrated by the data in *Figures 11.11 and 11.12*, are more serious; the occurrence of resistant strains requires an urgent approach for developing alternative protection procedures and products.

There is a need to re-examine the physiological factors which influence the condition of citrus fruits at harvest and the stresses which result in increased fungal infection. Ideally, post-harvest practices should try to minimize the activities of the storage rot pathogens while maintaining the vitality and disease resistance of the fruit.

These results of our studies on wound depth indicate the need to emphasize improved practices and more effective fungicide products.

Acknowledgement

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THERAPY AND NATURAL DEFENCES IN MASTITIS: I THE PHAGOCYtic DEFENCE OF THE UDDER

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Introduction

Mastitis is one of the major causes of loss to the dairy industry due to a combination of decreased production from affected quarters, cost of veterinary treatment, the discarding of milk from treated cows and early culling. Despite the many potential natural defence mechanisms contained in the udder, these often fail to control bacterial growth. Hibbitt and Hill (1977) reviewed the natural defence mechanisms of the mammary gland. The main emphasis of their work was the study of the basic proteins from the teat canal which form the first line of defence against invading bacteria. These basic proteins damaged bacterial cell walls and even though the bacteria were not always killed they were nevertheless rendered more susceptible to phagocytosis.

There has been much research effort since that time directed at defining the relative importance of the other antimicrobial mechanisms in the mammary gland. These include the nature and concentration of opsonins in the gland, the number of phagocytes present, the rate at which neutrophils (PMN) enter the gland after bacterial infection and the relative efficiency of PMN at bacterial phagocytosis and kill (variation among individuals). There is a paucity of data on the role of the macrophage in the udder and some difficulty in distinguishing mammary gland epithelial cells from macrophages (Lee, Wooding and Kemp, 1980). Macrophages obtained from the udder do phagocytose bacteria, but their bactericidal activity is much less than that of neutrophils. The role of these macrophages is nevertheless thought to be important and recent work (Craven, 1983) has shown that macrophages which have ingested bacteria release chemotaxins which could trigger the diapedesis of neutrophils into an infected udder.

When there is a rapid response by the bovine udder to invasive microorganisms, large numbers of PMN enter the gland and ingest and kill the organisms (Schalm, Lasmanis and Carroll, 1964; Jain, Schalm and Lasmanis, 1971; Hill, Shears and Hibbitt, 1978). *In vitro* techniques have been developed to measure the relative bactericidal activity of PMN from different cows and also to identify and measure the concentration of opsonins in serum and milk whey (Williams and Bunch, 1981; Williams and Hill, 1982).

Opsonic requirement of bovine PMN**SERUM AND WHEY FROM NORMAL COWS**

In the *in vitro* test of bacterial activity described herein, the final bacterial and PMN concentrations were, respectively, 1×10^6 per ml and 3 to 4×10^6 per ml in a final volume of 0.6 ml per roller tube. Opsonin concentrations varied as described and the tubes were rolled at 120 r.p.m. at 37°C.

There was a sufficiently high concentration of opsonin in bovine serum, even after dilution and complement inactivation, for PMN to phagocytose and kill over 90% of *Staphylococcus aureus* (strain M60) and an encapsulated strain (B117) and a non-encapsulated strain (P4) of *Escherichia coli* (Tables 12.1-12.3).

IgG from normal serum or whey failed to opsonize these bacteria (Table 12.1), but an IgM-rich fraction prepared after a combination of salt fractionation, anion exchange and gel filtration chromatography was opsonic to a final dilution of 1/3200 (*S. aureus*) and 1/400 (*E. coli* strain P4) (Williams and Hill, 1982). This represented most of the opsonic activity found in whole serum. However, the encapsulated strain of *E. coli* (B117) was found to be more resistant to phagocytosis and killing by PMN and required approxi-

Table 12.1 PERCENTAGE SURVIVAL OF 10^6 BACTERIA AFTER AGITATION FOR 2 h AT 37°C WITH 3×10^6 WASHED BLOOD NEUTROPHILS IN THE PRESENCE OF DIFFERENT TEST OPSONINS (TREATMENT)

<i>Treatment</i>	<i>Staphylococcus aureus</i> (M60)	<i>Escherichia coli</i> (P4)
Hanks' BSS	112	464
Pooled milk whey (5%)	1	4
Pooled heated serum (0.5%)	3	10
Serum IgG ₁	97	232
Serum IgG ₂	62	177
Serum IgM } 5 mg/ml	8	4

Source: from Williams and Hill (1982).

Table 12.2 PERCENTAGE SURVIVAL OF ENCAPSULATED AND NON-ENCAPSULATED STRAINS OF *Escherichia coli* AFTER 2 h AGITATION AT 37°C IN POOLED SERUM OR VARIOUS MILK WHEYS AND WASHED BOVINE NEUTROPHILS

<i>Strain</i>	<i>Serotype</i>	<i>Survival (%)</i>		
		Heated pooled serum	Pooled normal whey	Pooled newly calved whey
B117	08 K85	0.13	1010	1.9
B44	09 K30	0.04	1000	3.5
987	09 K103	0.05	800	1.8
Encapsulated mean		0.07	937	2.40
P4	032	0.18	0.04	0.02
B44	09	0.01	0.69	0.5
987	09	0.05	0.05	0.1
Non-encapsulated mean		0.08	0.26	0.21

Source: from Hill, Heneghan and Williams (1983).

mately 150 times as much serum opsonin to achieve the same degree of killing as *E. coli* (P4) in the *in vitro* test (Hill, Heneghan and Williams, 1983). Consequently, in view of the lower levels of IgM present in normal mid-lactation milk, the undiluted whey from all the cows tested failed to opsonize *E. coli* (B117) for phagocytosis. This resistance to opsonization by strain B117 was shared with all other encapsulated strains of *E. coli* tested (Tables 12.2 and 12.3). In contrast, early lactation pooled whey (5–10 days postpartum) was opsonic for all strains of *E. coli* (Table 12.2) and therefore severe *E. coli* mastitis which is more common in this early stage of lactation was not due to opsonic deficiency (Hill, Heneghan and Williams, 1983).

Table 12.3 RELATIVE OPSONIC ACTIVITY OF POOLED SERUM AND MID-LACTATION MILK WHEY AGAINST ENCAPSULATED AND NON-ENCAPSULATED STRAINS OF *Escherichia coli*

Strain of <i>Escherichia coli</i>	Opsonic titre ^a	
	Pooled serum	Mid-lactation whey
Encapsulated (n = 3)	1/26	estimated 1/1
Non-encapsulated (n = 3)	1/2000	1/82

Source: from Hill, Heneghan and Williams (1983).

^a Reciprocal of dilution of opsonin required to promote phagocytosis and death of 50% of *E. coli* by bovine PMN after 2 h agitation at 37°C.

SERUM FROM IMMUNIZED STEERS

Adult steers were injected subcutaneously in the neck at approximately monthly intervals with 10⁹ live bacteria and blood sampled after 16 weeks. Three steers were given *E. coli* (P4), 2 were given *E. coli* (B117) and a further 2 given *S. aureus* (M60). Serum samples were fractionated and each fraction tested at a concentration of 5 mg/ml for the presence of opsonins. The results are summarized in Table 12.4. No IgG opsonins were raised against *S. aureus* after homologous or heterologous immunization, when compared with values given in Table 12.1.

All animals injected with *E. coli* (P4 or B117) produced homologous opsonins in the IgG₂ fraction. The apparent loss of activity in the IgM fraction

Table 12.4 OPSONIC ACTIVITY IN THE FRACTIONATED SERUM OF STEERS IMMUNIZED SUBCUTANEOUSLY IN THE NECK WITH LIVE BACTERIA. GEOMETRIC MEAN PERCENTAGE OF SURVIVALS ($\pm$ S.D. OF THE LOG MEAN)

Serum fraction	<i>Escherichia coli</i> (P4) ^a n = 3	<i>Escherichia coli</i> (B117) ^a n = 2	<i>Staphylococcus aureus</i> (M60) ^a n = 2	<i>Staphylococcus aureus</i> (M60) ^b n = 5
IgG ₂	39.4 $\pm$ 0.46	13.7 $\pm$ 0.32	86.8 $\pm$ 0.04	79.7 $\pm$ 0.08
IgG ₁	193.0 $\pm$ 0.40	118.9 $\pm$ 0.17	100.6 $\pm$ 0.02	104.0 $\pm$ 0.11
IgM	474.0 $\pm$ 0.17	21.9 $\pm$ 0.20	45.2 $\pm$ 0.01	15.6 $\pm$ 0.31

^a Injected with and tested with homologous organisms.

^b Injected with *Escherichia coli* (P4) or (B117) and tested with *Staphylococcus aureus* (M60).

against *E. coli* (P4) may be the result of partial denaturation of IgM during its preparation.

EXPERIMENTAL INTRAMAMMARY INFECTION WITH *E. coli* (B117)

Escherichia coli (B117) was shown to be resistant to opsonization by normal mid-lactation milk whey, but infection of a single mammary quarter with 300 colony-forming units of this capsular strain stimulated a long-lasting opsonic activity (Table 12.5). Other strains of *E. coli* sharing a common capsular antigen (K85) were also opsonized by this immune whey. Although only one quarter of the udder was infected and the bacteria were naturally eliminated without antibiotic therapy, the three uninfected quarters of each udder all produced milk whey which was opsonic for strain B117 within 2 weeks of infection. This induced opsonic activity was stable to heating to 56°C (Hill *et al.*, 1983).

When opsonically active immune whey was fractionated, it was apparent that there was both an increase in the activity of IgM and the appearance of opsonic activity in the IgG₂ fraction (Table 12.6). However, this induced opsonic activity often failed to protect and the animals often became severely ill when experimentally infected with the homologous strain early in a subsequent lactation. This observation supports the view of Hill (1981) that the major problem associated with early lactation is not one of opsonic deficiency, but failure of rapid migration into the gland cistern. Further support to this

Table 12.5 THE DEVELOPMENT OF OPSONIC ACTIVITY IN MILK WHEY FROM EACH QUARTER OF 2 COWS FOLLOWING INTRAMAMMARY INFECTION WITH *Escherichia coli* (B117) IN THE LEFT FRONT GLAND ONLY

<i>Time of sampling</i>	<i>Geometric mean survival of bacteria (%)</i>	<i>Standard deviation (of log mean)</i>	<i>n</i>
Before infection	818.6	0.08	8
After infection (days 16 and 30)	0.17	0.17	16

Source: from Hill *et al.* (1983).

Table 12.6 OPSONIC ACTIVITY AGAINST *Escherichia coli* (B117) IN MILK WHEY AND FROM GLOBULIN FRACTIONS ISOLATED FROM MILK WHEY

	<i>Pre-infection</i>			<i>Post-infection</i>		
	<i>Geometric mean survival (%)</i>	<i>S.D. (of log)</i>	<i>n</i>	<i>Geometric mean survival (%)</i>	<i>S.D. (of log)</i>	<i>n</i>
Whey	819	0.08	8	0.17	0.17	16
IgG ₂	561	0.08	10	15.0	0.20	17
IgG ₁	509	0.20	6	103.0	0.36	14
IgM ^a	153	0.53	8	14.0	0.71	19

Source: from Hill *et al.* (1983)

^a The opsonic activity of both pre-infection and post-infection fractions of IgM were similar for *Staphylococcus aureus* (M60) and *Escherichia coli* (P4).

view is provided by the demonstration of the excess of opsonins to *S. aureus* (M60) and *E. coli* (P4) found in mid-lactation milk whey (Table 12.1).

Response of the udder to bacterial infection

MID-LACTATION

Experimental infections of the udder with serum-resistant strains of *E. coli* and *S. aureus*, or infusions of sterile culture filtrates of these organisms, generally stimulate a rapid infiltration of PMN into the gland by a mechanism which has yet to be elucidated. A clearer understanding of the early pathogenesis of *E. coli* infection of the bovine udder has resulted from the studies of Frost, Hill and Brooker (1980). Localized epithelial necrosis resulted in lesion formation in the teat and lactiferous sinuses, through which the PMN migrate onto the surface and then into the lumen of the gland (see Figures 12.1–12.3). The result of this rapid PMN mobilization often results in the elimination of *E. coli* before clinical signs of the disease are evident. In contrast, the outcome of *S. aureus* infection may be a chronic infection.

EARLY LACTATION

The migration of PMN into the cistern of the gland is often delayed at this stage of lactation (Hill, 1981). This allows *E. coli* multiplication to proceed to higher numbers, resulting in correspondingly higher toxin concentrations leading to a severely ill animal. In some high-yielding cows in early lactation, PMN migration totally fails, resulting in severe epithelial necrosis and haemorrhagia in the underlying tissue (Figures 12.1 and 12.4), systemic endotoxaemia and death of the animal.

Variation among individuals in the bactericidal activity of their PMN

The defensive role of PMN against both coliform and staphylococcal infection in the udder was shown clearly when their numbers were reduced by administering anti-leucocyte serum (Jain, Schalm and Lasmanis, 1971; Schalm, Lasmanis and Jain, 1976). The bactericidal activity of PMN was reduced in milk, mainly due to degranulation following ingestion of fat globules (Paape and Wergin, 1977) and casein (Russell, Brooker and Reiter, 1977). Naidu and Newbould (1973) postulated that the poor phagocytic competence of mammary gland PMN was due partially to low energy reserves of glycogen in these cells. Newbould (1973) further showed that milk-derived PMN from different cows varied in their *in vitro* phagocytic competence for staphylococci. The addition of glucose enhanced *in vitro* phagocytosis, but the effect was less marked with the PMN from cows of lower phagocytic activity. Paape, Pearson and Schultze (1978) also showed significant variation in the overall bactericidal activity of PMN isolated from milk, and it appeared that the prevalence of clinical mastitis was greater in these cows whose PMN had a lower capacity for phagocytosing staphylococci.

PMN isolated from bovine blood have also been shown to vary widely

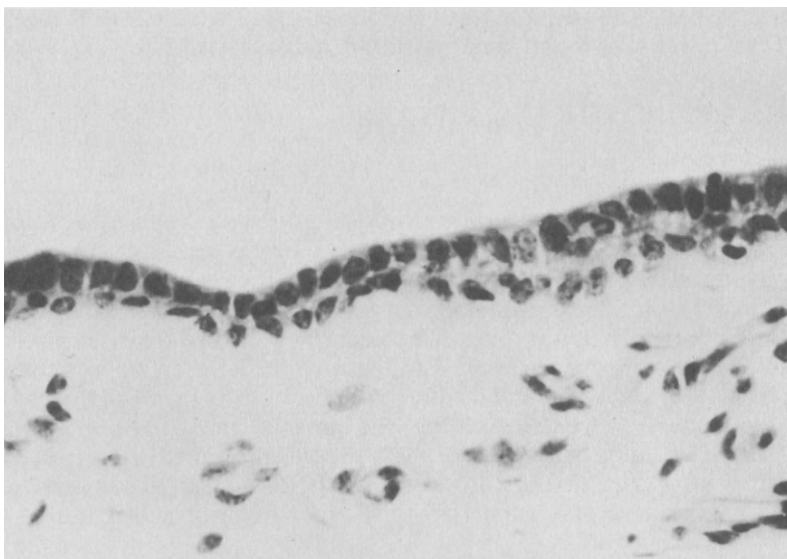


Figure 12.1 Normal lactiferous sinus showing two-cell thick epithelium and underlying tissue devoid of neutrophils (haematoxylin and eosin, H and E, stain $\times 380$)

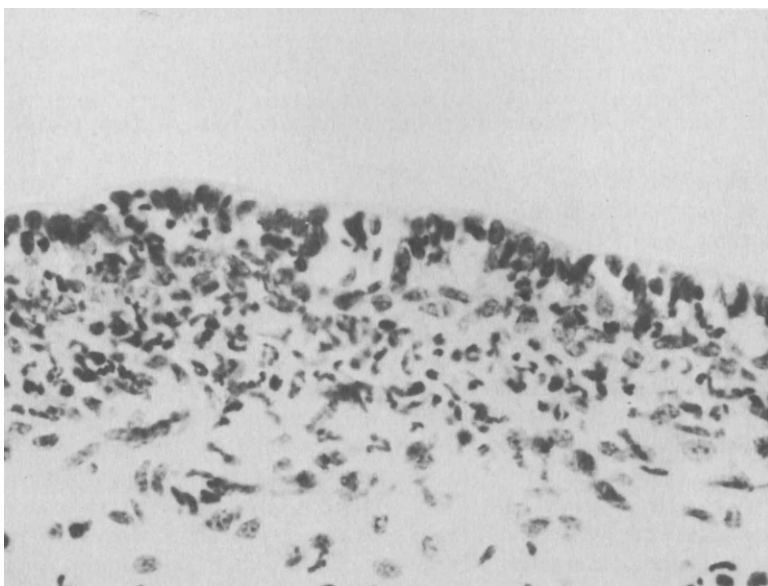


Figure 12.2 Lactiferous sinus of a cow in mid-lactation 10 h after infection with *Escherichia coli*, showing sub-epithelial neutrophilia (H and E $\times 380$)

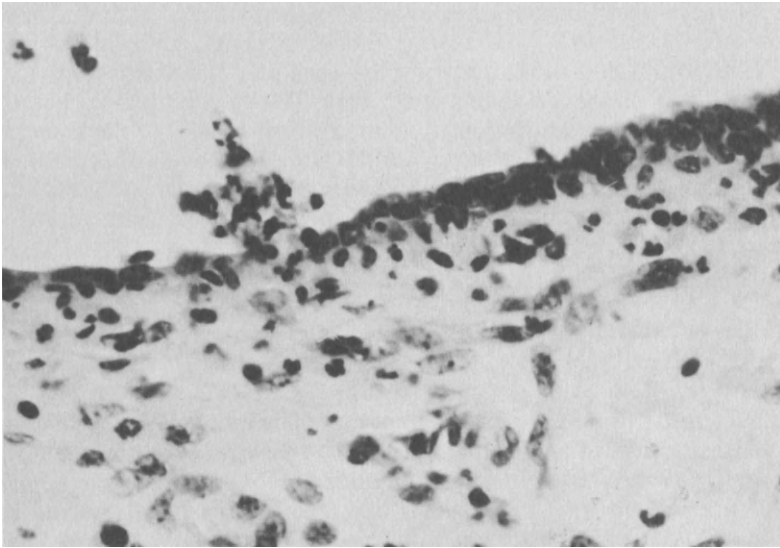


Figure 12.3 Lactiferous sinus of a cow in mid-lactation 14 h after infection with *Escherichia coli*, showing movement of sub-epithelial PMN through a localized lesion into the lumen of the gland (H and E $\times 380$)

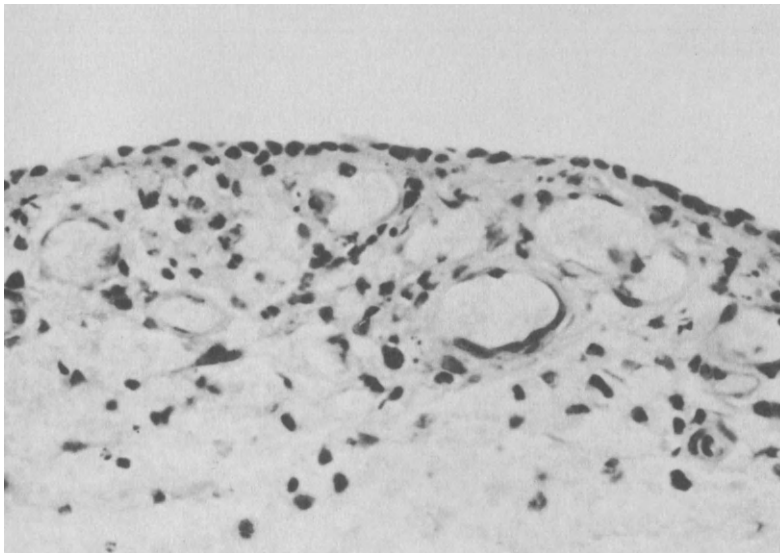


Figure 12.4 Lactiferous sinus of a cow 36 h after infection at parturition with *Escherichia coli*, showing loss of both layers of epithelial cells, dilation of capillaries, severe haemorrhage into the tissue and complete absence of neutrophilia (H and E $\times 300$)

among cows in their bactericidal activity against *E. coli* and *S. aureus* (Williams and Bunch, 1981) and against *S. agalactiae* (Mackie, Pollock and Logan, 1982). Bulls and calves show similar variation in PMN bactericidal activity against *S. aureus* (Williams *et al.*, 1984). When 0.5% pooled heated bovine serum was used for opsonization, an average of 90% of the bacteria were killed by 2 h. There was a tendency for PMN with low overall bactericidal activity to show higher numbers of PMN-associated bacterial survivors and this suggested both a low rate of phagocytosis and a reduced ability of PMN to kill ingested intracellular bacteria.

Association between the rate of phagocytosis and intracellular killing of bacteria within PMN

The 32 bulls tested for bactericidal activity of their blood PMN (Williams *et al.*, 1984) were ranked and bulls 1-5 (high PMN activity) and 28-32 (low PMN activity) were re-tested for total bactericidal activity, total extracellular bacterial survival and total intracellular bacterial survival after removal of extracellular *S. aureus* by treatment with lysostaphin (Easmon, Lanyon and Cole, 1978).

Table 12.7 THE ASSOCIATION BETWEEN RATE OF PHAGOCYTOSIS AND INTRACELLULAR KILLING OF BACTERIA WITHIN NEUTROPHILS

Relative bactericidal activity	Geometric mean (%) <i>Staphylococcus aureus</i> $\pm$ S.D. ^b		
	Total survivors	Extracellular (supernatant)	Intracellular survivors
High ^a	3.4 $\pm$ 0.11	1.7 $\pm$ 0.19	0.55 $\pm$ 0.14
Low ^a	15.4 $\pm$ 0.17	6.0 $\pm$ 0.19	1.28 $\pm$ 0.08

^a 5 bulls in each group.

^b S.D. of log % survival.

Table 12.8 RANKING OF HIGH- AND LOW-ACTIVITY BULLS FOR INTRACELLULAR KILLING OF *Staphylococcus aureus*, TOTAL BACTERICIDAL ACTIVITY AND RELATIVE CHEMOTAXIS OF PMN TOWARDS A CHEMO-ATTRACTANT

PMN activity	Killing of internalized bacteria	Total bactericidal activity	Chemotaxis
High	H1	H1	
	H2	H5	H2
	H3	H2	L7
	H4	H3	L9
	H5	H4	
			H1, H5, L6
Low	L6	L6	
	L7	L9	H3
	L8	L7	L8
	L9	L8	L10
	L10	L10	H4

The group of bulls with high PMN activity (low total bacterial survivors) had low numbers of extracellular *S. aureus* (in the supernatant) and low numbers of surviving *S. aureus* within PMN (Table 12.7). These 10 bulls were also ranked according to the relative chemotactic activity of their isolated PMN under agarose towards a standard chemo-attractant (fresh serum). The chemotaxis assay was a modification by Craven (1983) of the technique of Nelson, Quie and Simmons (1975). Table 12.8 shows that there is no apparent relationship between chemotaxis of bovine PMN and their ability to phagocytose and kill *S. aureus*, but the association between ability to kill ingested bacteria and overall bactericidal activity (mainly a measure of the rate of phagocytosis) is very high.

Summary

The bovine udder contains a number of potential antimicrobial mechanisms, but these often fail to control bacterial growth. There are sufficient opsonins in the gland to opsonize *S. aureus* and non-capsular *E. coli*. IgM is the major opsonin for bacterial phagocytosis by neutrophils, although IgG opsonins may also be produced after experimental injection or infection. There are large differences among cows, bulls and calves in the ability of their neutrophils to phagocytose bacteria and this is also correlated with their ability to kill the organisms after ingestion. The ability of the neutrophils to enter an infected mammary gland rapidly and in large numbers is thought to be of even greater importance in the control of infection. The udders of cows which fail to respond early are often damaged by bacterial toxins.

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THERAPY AND NATURAL DEFENCES IN MASTITIS: II INTERACTION OF ANTIBIOTICS AND PHAGOCYTES IN MASTITIS THERAPY

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Introduction—a conflict of strategies?

The concept of chemotherapy, as first proposed by Ehrlich (1906), envisaged the treatment of infections by chemicals which showed a selective toxicity for invading microorganisms. However, the early achievements of antimicrobial chemotherapy were slight and the discovery by Metchnikoff of the role of phagocytes in defence against infection was given greater prominence through the work of Wright and others. Thus, Shaw was prompted to make his well-known observation that the only scientific treatment for all diseases was to 'stimulate the phagocytes. Drugs are a delusion'. This popular view was eclipsed by chance observations made, ironically, in Wright's laboratory by Fleming (1929) which led to the discovery of penicillin. The subsequent search for other antibiotics and their successful isolation and introduction into medical and veterinary usage seemed to confirm Ehrlich's original prediction.

Early euphoria has now been tempered by some 40 years of clinical experience with antibiotics. In veterinary medicine the optimism with which penicillin was introduced was largely justified in the treatment of bovine streptococcal mastitis, but was confounded by limited success in treating staphylococcal infections (Lovell, 1946). Despite a greatly increased armoury of antibacterial agents available today, there have been few real advances in the therapy of mastitis (Mercer *et al.*, 1976) and cure rates against staphylococcal mastitis, in particular, remain disappointing.

Low bacteriological cure rates, emergence of antibiotic resistance and concern about antibiotic residues in milk have all led to pressure to reduce antibiotic usage during lactation and to a renewal of interest in means of stimulating and exploiting the phagocytic defence mechanisms of the udder. However, although work on immunization against mastitis is progressing (Pankey, 1980), fundamental objections to this approach still remain (Anderson, 1978; 1982). In this chapter we describe how phagocytes and antibiotics may interact synergistically or antagonistically in mastitis therapy and we consider the prospects for improved therapeutic strategies in which antibiotics may augment natural defences.

Antibiotics and host defences

The aim of therapy is to achieve and maintain active levels of the antibacterial agent within the infected host tissues until sterility is attained. Antibacterial activity is routinely measured *in vitro* under conditions which often bear little relationship to those *in vivo*, and emphasis is traditionally placed on the need to maintain antibiotic levels in excess of the minimum inhibitory concentration (MIC) or, for bactericidal agents, the minimum bactericidal concentration (MBC). However, it was realized in early studies that sub-MIC concentrations of antibiotics could produce morphological changes in bacteria (Gardner, 1940) and such concentrations may also diminish both the rate and final amount of bacterial growth (Rolinson, 1977). Recent reports suggest that 'sub-inhibitory' levels of antibiotics may have beneficial therapeutic effects *in vivo* (for reviews, see Ahlstedt, 1981, and Atkinson and Amaral, 1982). On the other hand, bacterial susceptibility to antibiotics *in vitro* does not guarantee a successful outcome of therapy; host tissue reactions may also impede antibiotic action.

Many studies have considered the interactions of antibiotics, bacteria and various components of the immune system. The results are confusing and often contradictory. Antibiotics may, in addition to their effect on bacteria, exert a direct effect on any or all stages of phagocytic cell activity, i.e. chemotaxis, opsonization, ingestion, oxidative metabolism and intracellular killing, by either increasing or decreasing the function. Furthermore, bacteria which have first been sub-lethally damaged by antibiotics may be rendered more (rarely less) susceptible to the activity of phagocytes or to humoral factors such as complement or lysozyme. Alternatively, bacteria which survive intracellularly within phagocytes may be protected from subsequent killing by antibiotics (for a review of antibiotic-neutrophil interactions, see Yourtee and Root, 1982). Other reported consequences of sub-lethal antibiotic exposure which may influence the progress of infection include increased susceptibility to agglutinating antibodies, decreased bacterial adherence to host cells and increased toxin production by bacteria (Atkinson and Amaral, 1982). In our studies on staphylococcal mastitis, we have attempted to evaluate the relative importance of such interactions in determining the response to therapy.

Phagocytes and mastitis therapy

Bovine mastitis is a disease which is defined in terms of the local inflammatory response. Since many of the clinical signs of inflammation arise directly from the neutrophil influx within the gland (Jain, 1976), recognition of the symptoms of mastitis by the herdsman coincides with the defence reaction occurring in the udder. Antibiotics are thus administered at some variable time after the initial encounter between bacteria and phagocytes. In peracute mastitis, where the cow fails to mobilize sufficient neutrophils (PMN) into the affected quarter, unrestricted rapid bacterial growth may occur before mastitis is recognized. In such a case very high bacterial numbers and relatively few phagocytes may be present at the time of therapy and host defences may be further compromised by bacterial toxin production. At the other

extreme, in cases of chronic mastitis the disease may remain sub-clinical and undetected for long periods, with a balance being maintained between bacteria and phagocytes. In these cows, antibiotics will usually be given during a 'flare-up' of clinical disease and some interaction between antibiotics and the phagocytic defences may then be expected. Bovine staphylococcal mastitis is typically chronic. Peracute, gangrenous mastitis is relatively infrequent.

In our studies on therapy we have used an experimental mouse model of mastitis which was originally described by Chandler (1970). Lactating mice are inoculated via the teat duct into the fourth mammary gland on either side (Anderson, 1976). The typical reaction in the mouse to an intramammary inoculation of an α -toxin-producing strain of *Staphylococcus aureus* is acute mastitis. However, a reaction resembling chronic mastitis can be obtained in glands that have been pretreated with endotoxin (Anderson, 1977). The validity of this mouse mastitis model when extrapolated to the cow has been demonstrated (Anderson and Heneghan, 1979).

ACUTE MASTITIS

Inoculation of c. 10^6 cfu (colony forming units) *S. aureus* strain M60 into normal lactating mouse mammary glands was followed by rapid bacterial multiplication, production of α -toxin and liquefactive necrosis. Although some PMN infiltrated the glands within 6 h of infection, they were unable to restrain bacterial growth and, in the absence of treatment, infection was fatal within 24 h. Intramammary administration of 1 mg sodium cloxacillin simultaneously with, or soon after, infection prevented clinical disease and reduced bacterial numbers, whereas treatment of more advanced disease was unsuccessful (Figure 13.1). Autoradiographic studies using radiolabelled cloxacillin indicated that, although distribution of antibiotic within such mastitic glands was somewhat impaired, inhibitory concentrations were achieved throughout diseased tissues and were maintained for the duration of the tests (Craven and Anderson, 1982a). Since penicillins exert their bactericidal action on dividing bacteria and are ineffective against stationary phase organisms (Figure 13.2), the failure of therapy in the later stages of the acute reaction may be attributed solely to the high bacterial numbers present and their decelerated rate of multiplication. The necrosis resulting from toxin production may have prevented any useful host defence reaction from developing in the later stages. Reduction of the initial inoculum to only c. 10^2 organisms per mouse mammary gland merely extended the *in vivo* growth phase of the reaction by a few hours (Craven, 1981). Thus, therapy was only of value in the first few hours following infection before the microbiological and pathological changes reached a refractory stage. In the cow, such acute reactions may well go unrecognized until too late.

CHRONIC MASTITIS

Infusion of 25 μ g endotoxin into a normal lactating mouse mammary gland induced a neutrophil infiltration of the milk duct system. When 10^7 cfu *S. aureus* strain M60 were inoculated 6 h after endotoxin, the PMN failed to

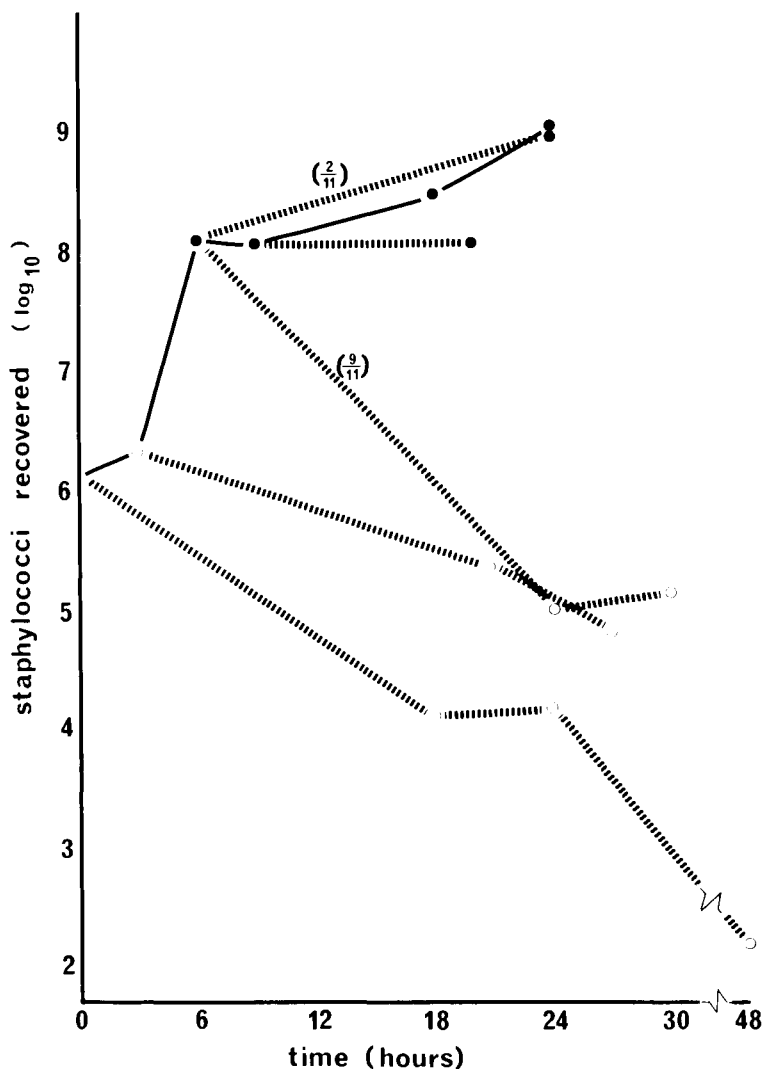


Figure 13.1 Effect of intramammary cloxacillin on acute staphylococcal mastitis in mice. Each point shows the mean bacterial numbers per gland from 4 or more mice. Solid line shows growth of *Staphylococcus aureus*, strain M60, in untreated glands. 1 mg cloxacillin was administered at different times after infection and the response to therapy is shown by broken lines (of 11 mice treated 6 h after infection, 2 failed to respond, as indicated by figures in brackets). Solid symbols show groups in which haemolysins were detected in mammary gland homogenates (from Craven and Anderson, 1982a, by courtesy of *Journal of Comparative Pathology*. Copyright: Academic Press Inc. (London) Ltd)

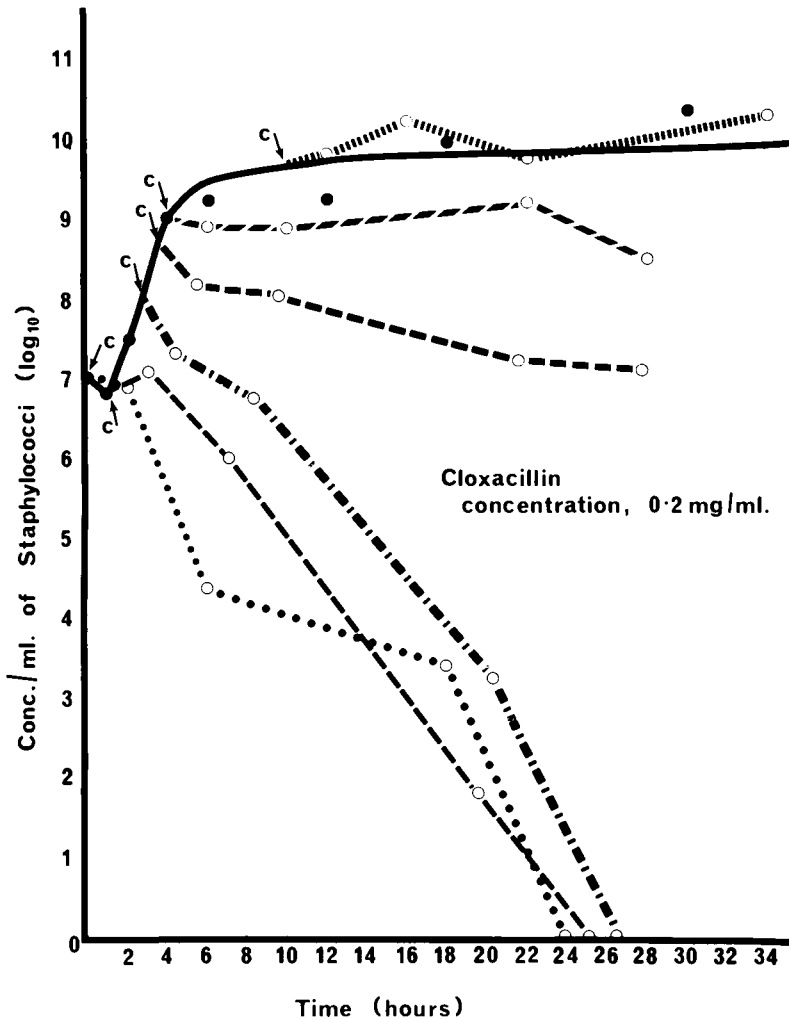


Figure 13.2 Action of cloxacillin on *Staphylococcus aureus* growing *in vitro*. *S. aureus*, strain M60, was grown in Hartley's broth at 37°C. Cloxacillin was added to 200 µg/ml, at points marked with arrows, and bacterial viability in these samples was monitored thereafter, as shown by broken lines (from Craven, 1981)

eliminate the staphylococci, but an equilibrium was established with the mice remaining clinically normal. Subsequent intramammary administration of 1 mg cloxacillin 48 h after infection had no effect on the numbers of bacteria surviving within the glands (Anderson, 1977). Distribution of cloxacillin within such glands, as assessed by an autoradiographic technique, showed considerable impairment of diffusion of antibiotic from the milk ducts to the parenchyma. Nevertheless, inhibitory levels (c. $10 \times \text{MIC}$) were attained throughout the tissue (Craven, 1981). Electron microscopy revealed that most staphylococci were intracellular within PMN (Figure 13.3), macrophages and also alveolar epithelial cells. When suspensions of cells containing staphylo-



Figure 13.3 Mouse mammary tissue 48 h after infection with *Staphylococcus aureus*, strain M60. The neutrophil (N) contains staphylococci which are in various stages of degeneration (numerically indicated); E, epithelial cell. $\times 12\,800$ (from Craven and Anderson, 1979, by courtesy of *British Journal of Experimental Pathology*)

cocci were prepared from chronically infected glands and incubated *in vitro*, a reduction in bacterial numbers occurred, but this reduction was greater when the somatic cells were first disrupted with ultrasound, implying that the intracellular location of the bacteria afforded protection against the antibiotic (Craven and Anderson, 1979).

Antibiotic-phagocyte antagonism

Further *in vitro* studies were designed to examine the survival of staphylococci within bovine phagocytes in the presence of antibiotics. It was found that the small proportion of staphylococci that somehow resist intracellular killing and survive within bovine PMN, were subsequently protected from the bactericidal action of cloxacillin, provided that the PMN remained intact. However, by chance, it was noticed that when staphylococci were released from inside PMN following exposure to extracellular cloxacillin, the bacteria had become significantly more sensitive to killing by the cell-wall lytic enzyme lysostaphin (Craven and Anderson, 1980a). Similar effects have also been seen with *S. aureus* surviving inside bovine mammary macrophages (Table

13.1). This increase in sensitivity to muralytic enzymes is probably indicative of sub-lethal cell wall damage by penicillins, cell walls from penicillin-treated *S. aureus* being more sensitive to endogenous muralysin activity (Takebe *et al.*, 1970). Thus, it was apparent that the cloxacillin was producing a sub-lethal effect on the intracellular staphylococci. These effects were seen with $10 \times$ MIC extracellular cloxacillin concentrations, and a further 40-fold increase in concentration still failed to reduce intracellular bacterial viability, indicating strong protection against the bactericidal action of the drug.

Table 13.1 SENSITIVITY TO LYSOSTAPHIN OF *Staphylococcus aureus* AFTER EXPOSURE TO ANTIBIOTICS WHILE INSIDE MACROPHAGES

<i>S. aureus</i> obtained from inside macrophages after 20 h incubation in medium containing:	Percentage survival ^a of <i>S. aureus</i> diluted in lysostaphin (0.15 µg/ml)			
	In p.b.s. pH 6.8:		In HBSS pH 7.6:	
	strain M60 ^b	strain Oxford ^c	strain M60 ^b	strain Oxford ^c
No antibiotic (but lysostaphin, 5 µg/ml, to remove extracellular bacteria)	52.4 (± 11.0)	6.72	50.3 (± 13.37)	10.9
Cloxacillin (10 µg/ml)	0.53 (± 0.21)	0.8	0.59 (± 0.06)	0.78

^a Values represent: (Viable count in 0.15 µg/ml lysostaphin, pH 6.8)/(Viable count in p.b.s., pH 6.8) $\times 100$ (or a similar test using HBSS pH 7.6 as the diluent).

^b Mean of 3 ($\pm$ standard error of mean).

^c Results from single experiment.

The protection of staphylococci within phagocytes from antibiotic action is well established (Yourtee and Root, 1982), but to what extent antibiotics are able to penetrate phagocytic cells is more controversial. Early reports, based on the observation that intracellular staphylococci were not killed, suggested that penicillins could not penetrate somatic cells (e.g. Holmes *et al.*, 1966). This view was supported by others, who found that penicillin penetrated phagocytic cells only poorly (Mandell, 1973; Johnson *et al.*, 1980; Prokesch and Hand, 1982; Jacobs *et al.*, 1982). On the other hand, there are numerous reports that penicillins do penetrate phagocytes and affect intracellular bacteria (Showacre *et al.*, 1960; Cole and Brostoff, 1975; Veale *et al.*, 1976; 1979; Lowrie, Aber and Carrol, 1979).

The suggestion has been made that live intracellular bacteria may exert a toxic effect on the host phagocytic cells and thus induce an increase in permeability to antibiotics (Frost *et al.*, 1972; Veale *et al.*, 1979). It has also been suggested that activation of phagocytes may make them more permeable (Cole and Brostoff, 1975). This may be of relevance since many 'negative' studies have examined the penetration of penicillins into non-infected phagocytes. We agree with the conclusion of Yourtee and Root (1982) that penicillin enters cells, but that its activity against intracellular bacteria can be easily demonstrated only against bacteria that can multiply within cells. No evidence of intracellular multiplication was found for staphylococci surviving within bovine PMN (Craven, 1981) and it was concluded that the relative 'dormancy' of the intracellular organisms was the basis of their resistance to killing by cloxacillin in *in vitro* tests and perhaps also in chronic mastitis.

The role of pH in intracellular survival

An alternative and increasingly attractive explanation is that the pH of the phagosome may provide the 'shield' against killing by penicillins. Using yeast cells stained with indicator dyes, it was estimated that the pH of a substantial proportion of phagosomes of bovine PMN fell to pH 5.4 or lower and these

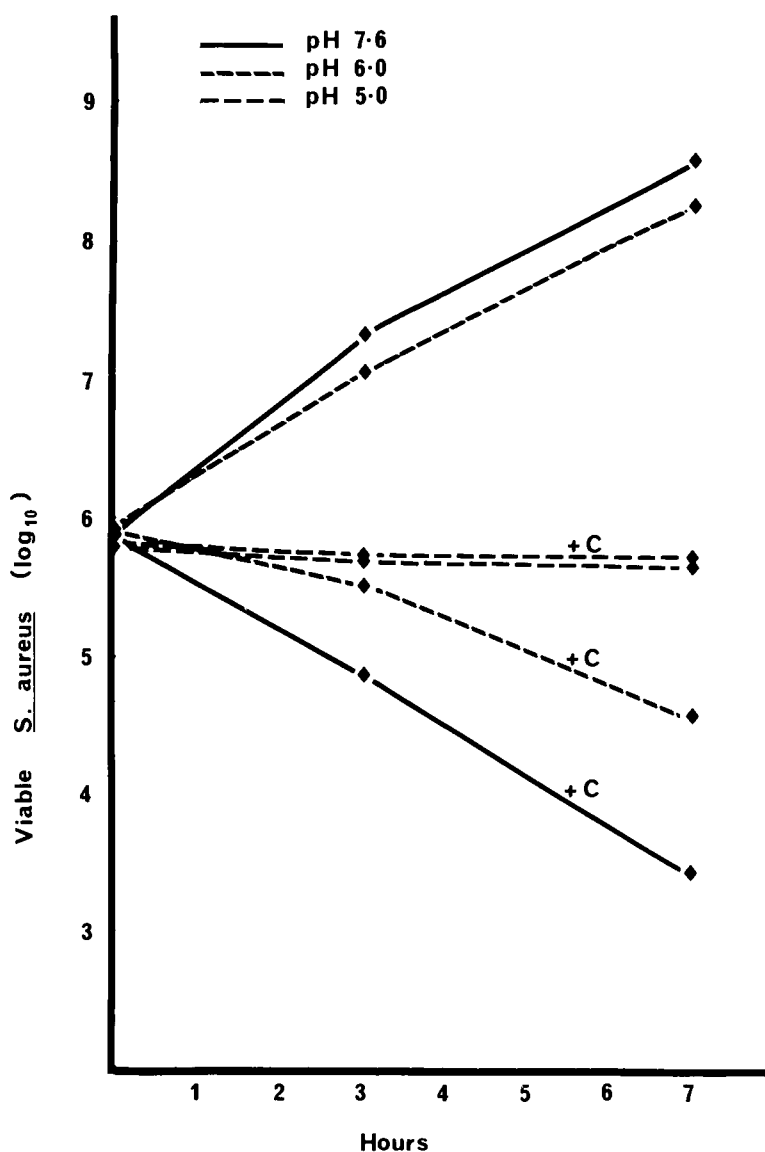


Figure 13.4 Growth of *Staphylococcus aureus*, strain Oxford, *in vitro* and killing by cloxacillin in broth buffered at different pH levels. + C = samples containing 5 µg of cloxacillin per ml. Each point is the mean of two or more observations (from Craven and Anderson, 1982b, by courtesy of *Antimicrobial Agents and Chemotherapy*)

levels were maintained over 2 h incubation periods (Craven, 1981). We then examined the effects of cloxacillin on *S. aureus* in broth cultures buffered at different pH levels (Figure 13.4). It was found that growth and killing of *S. aureus* by cloxacillin were most rapid at pH 7.6, whereas at pH 6.0, although growth was slightly reduced, the reduction in the bactericidal effect of cloxacillin was more marked.

This protection from killing by penicillins at low pH has been described as pH-dependent penicillin tolerance (Goodell, Lopez and Tomasz, 1976). It appears that bacterial autolytic enzymes are responsible for the final lethal lysis which results from penicillin exposure; at a critical pH level these autolysins are inhibited. Bacteria may still be able to grow under these conditions, but the effect of added penicillin is merely bacteriostatic.

At even lower pH levels, bacterial growth is itself inhibited. This was seen with *S. aureus* at pH 5.0 (Figure 13.4). Since the estimated pH within bovine PMN phagosomes was in the range over which *S. aureus* showed tolerance to cloxacillin, and since intracellular staphylococci have an increased sensitivity to lysostaphin following cloxacillin exposure, we tested the lysostaphin sensitivity of the broth-growth bacteria that had been protected from killing by cloxacillin by pH alone. It was found that at the pH level that protected the staphylococci from killing by cloxacillin (pH 6.0, 5.8), they nevertheless sustained cell-wall damage and showed an increase in sensitivity to lysostaphin (Table 13.2). Thus, observations on the effects of cloxacillin on intracellular *S. aureus* may be entirely accounted for by the low pH of the phagocytic vacuoles (Craven and Anderson, 1982b). The growth-limiting effects of low phagolysosomal pH were similarly proposed as the basis of protection of staphylococci within rabbit PMN from extracellular penicillin (Lam and Mathison, 1982).

It is worth recalling that the intracellular staphylococci which are protected from killing by penicillins are also those that have resisted the killing mechanisms of the PMN. However, such organisms do not appear to constitute a more resistant sub-population. Recent work with human PMN from patients with chronic granulomatous disease suggests that the greater survival of *S. aureus* within such cells than in normal PMN is entirely due to an unusually rapid and extensive fall in pH within the phagosomes which impairs the

Table 13.2 SENSITIVITY TO LYSOSTAPHIN OF *Staphylococcus aureus* GROWN IN BROTH AT DIFFERENT pH LEVELS

	Percentage survival ^a of <i>S. aureus</i> diluted in 0.15 µg/ml lysostaphin at pH 6.8, following incubation in broth at pH:							
	Oxford				M60			
	7.6	6.0	5.8	5.0	7.6	6.0	5.8	5.0
Untreated control <i>S. aureus</i> grown in medium for 5 h	83	71	84	87	114	65	110	104
<i>S. aureus</i> incubated with 2.5 µg/ml cloxacillin for 5 h (clox. inactivated at time of testing)	0	1	18	97	2	13	71	77

Source: from Craven and Anderson (1982b), by courtesy of *Antimicrobial Agents and Chemotherapy*.

^a Values represent: (Viable count in 0.15 µg/ml lysostaphin, pH 6.8)/(Viable count in phosphate buffered saline, pH 6.8) × 100.

killing and digestion of the intracellular bacteria (Segal *et al.*, 1981; 1982). It is thus tempting to speculate that the small proportion of viable intracellular *S. aureus* within normal bovine PMN might similarly owe their survival to the rapid acidification of the phagosome.

To investigate this hypothesis a series of phagocytic tests was carried out in which bovine PMN were mixed with *S. aureus* and opsonins in standardized ratios, in media of differing pH levels (Figure 13.5). Analysis of the findings showed that variation in pH of the media did not affect cell viability and had no significant effect on phagocytosis (ingestion) of *S. aureus*, but that pH had a highly significant effect on intracellular survival. This varied from 1% or less survival at pH 7.5, rising to 15% at pH 5.5 (N. Craven and M.R. Williams, unpublished observations). During phagocytosis by PMN, degranulation may commence before the vacuole is completely sealed from the exterior. Low extracellular pH may therefore have accelerated the acidification of the phagosome, promoting greater intracellular survival of staphylococci. However, it is also possible that the low pH of the medium had a detrimental effect on some unrecognized aspect of PMN function which inhibited intracellular killing.

On the basis of the above findings and in terms of pH alone, about four times more staphylococci might be expected to survive intracellularly in PMN suspended in normal milk (pH 6.6) than in PMN suspended in medium at the

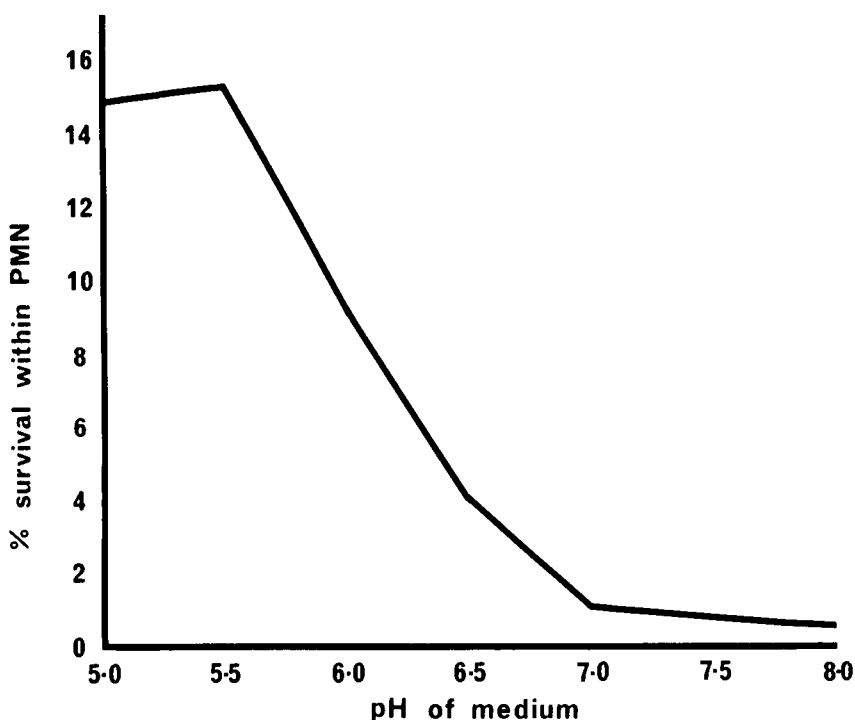


Figure 13.5 The mean percentage intracellular survival of *Staphylococcus aureus*, strain M60, is shown, after 2 h incubation with bovine PMN in Hanks' solution buffered at different pH levels

more usual 'physiological' pH of 7.5 or thereabouts. This observation is of interest since it is known that PMN suspended in milk have a reduced bactericidal activity when compared with those isolated from blood and suspended in tissue culture media. The bactericidal deficiency of milk PMN has been attributed to phagocytosis of casein (Russell, Brooker and Reiter, 1976) and/or milk fat globules (Reinitz, Paape and Mather, 1982). The latter authors demonstrated that ingestion of fat droplets by bovine PMN inhibited the pH depression within the phagosomes. They speculated further that *reduced* acidification of the phagosome might be the basis of the impaired bacterial killing. Our own findings would suggest the contrary, in that failure to acidify phagosomes should *favour* killing of *S. aureus*. It seems likely, therefore, that the inhibitory effects of ingested milk fat droplets on bacterial killing are independent of their effect on phagosomal pH.

Antibiotic-phagocyte synergism

Using an *in vitro* system where *S. aureus* survived within bovine PMN and extracellular bacteria were removed with lysostaphin, a wide range of antibiotics was screened for intracellular bactericidal activity (Craven and Anderson, 1980b; 1983). Each antibiotic was tested at the arbitrary concentration of $10 \times \text{MIC}$. The majority of antibiotics tested showed no activity. Some failed to kill intracellular staphylococci, but increased their sensitivity to lysostaphin (the cell-wall active antibiotics). A few showed some true intracellular bactericidal action (Table 13.3). Substantial intracellular bactericidal activity was shown by rifampicin and rifamycin-SV, even at levels in slight excess of the MIC. Rifampicin may be concentrated within phagocytic cells (Mandell, 1973) and is bactericidal for *S. aureus* at low pH (Craven, 1981). It binds to bacterial DNA-dependent RNA polymerase and stops protein synthesis and also DNA synthesis after one round of replication has been completed (Riva and Silvestri, 1972). Its bactericidal action is thus independent of bacterial cell wall synthesis. These properties undoubtedly contribute to its intracellular bactericidal efficacy.

In the model of chronic mastitis in the mouse, where intracellular staphylococci represent the major therapeutic problem, intramammary therapy with rifampicin was effective in reducing the number of viable bacteria. However,

Table 13.3 ANTIBIOTIC^a ACTIVITY AGAINST INTRACELLULAR *Staphylococcus aureus*

No activity	No killing but increased sensitivity to lytic enzymes	Slight killing	Good killing
Gentamicin	Cloxacillin	Clindamycin	Rifampicin
Streptomycin	Flucloxacillin	Fusidate	Rifamycin-SV
Chloramphenicol	Nafcillin	Neomycin	
Erythromycin	Amoxycillin		
Trimethoprim + sulphadoxine	Cephadrine		
Novobiocin	Cefoperazone		
Nitrofurazone	Vancomycin		

^a All used at $10 \times \text{MIC}$.

in the early stages of acute mastitis, when bacterial multiplication was rapid, rifampicin-resistant bacteria emerged. Resistance to rifampicin is easily acquired through a single step mutation. Development of resistance was prevented by the use of a combination of rifampicin with cloxacillin. This combination showed neither antagonism nor synergy *in vitro*, but was more effective than either antibiotic alone in acute mastitis and killed *S. aureus* in the chronic infection as effectively as rifampicin alone (Craven and Anderson, 1981). Therapy of chronic mastitis in mice with rifamycin-SV was significantly less effective than was rifampicin, although they were equally active at killing intraleucocytic staphylococci *in vitro*. This *in vivo* difference may reflect their different pharmacokinetic properties (Craven and Anderson, 1983).

Although staphylococci surviving within phagocytes may be protected from subsequent killing by penicillins, a somewhat paradoxical effect was noticed when the bacteria were exposed to cloxacillin prior to adding to the phagocytes. It was found that the cloxacillin-treated staphylococci had been rendered more susceptible to killing by bovine PMN (Craven and Anderson, 1980b). It has been suggested that semi-synthetic penicillins may differ in their ability to enhance staphylococcal susceptibility to phagocytes and enzymic bacteriolysis. Pre-treatment with nafcillin was found to be more effective than other penicillins in augmenting phagocytosis of *S. aureus* by mouse peritoneal macrophages (Friedman and Warren, 1974) and in enhancing bacteriolysis by lysozyme and trypsin (Warren and Gray, 1965). However, nafcillin, cloxacillin and penicillin G were all equally effective at increasing *in vitro* susceptibility of *S. aureus* to enzyme mixtures from leucocytes (Efrati *et al.*, 1976). Since bovine PMN constitute the major defensive barrier against bacterial infection in the udder and lysozyme is present only in small amounts (Vakil *et al.*, 1969), we examined the effects of pre-treating *S. aureus* with amoxycillin, cloxacillin or nafcillin on susceptibility to killing by bovine PMN (Craven, Williams and Anderson, 1982). Staphylococci were pre-treated by either growing overnight broth cultures in the presence of 1/4 MIC penicillin or by a 1 h exposure of a growing culture to 10 × MIC penicillin. All penicillins were found to increase the susceptibility of *S. aureus* to bovine PMN (Tables 13.4 and 13.5). Cloxacillin was significantly more effective than nafcillin or amoxycillin in some tests and equally effective in others.

Table 13.4 KILLING BY BOVINE PMN OF *Staphylococcus aureus* PRE-TREATED WITH PENICILLINS, AFTER 18 h GROWTH IN 1/4 MIC (0.1 µg/ml) PENICILLINS

	<i>Oxford</i>		<i>M60</i>	
	30 min	120 min	30 min	120 min
Amoxycillin	42 ± 8 ^a	115 ± 41	86 ± 7	94 ± 9
Cloxacillin	26 ± 9 ^a	55 ± 18 ^b	48 ± 9 ^a	72 ± 9 ^b
Nafcillin	42 ± 11 ^a	95 ± 30	62 ± 13 ^b	62 ± 18

Source: from Craven, Williams and Anderson (1982), by courtesy of *Comparative Immunology, Microbiology and Infectious Diseases*.

Mean of 6 (± SEM).

Table shows mean survival of penicillin-treated *S. aureus* as a percentage of control (untreated) survival.

From paired sample Student's 't' tests.

^a Significantly fewer survivors than in control, $P < 0.01$.

^b Significantly fewer survivors than in control, $P < 0.05$.

Table 13.5 KILLING BY BOVINE PMN OF *Staphylococcus aureus* PRE-TREATED WITH PENICILLINS, AFTER 45 min EXPOSURE TO 10 × MIC (4 µg/ml) PENICILLINS

	Oxford		M60	
	30 min	120 min	30 min	120 min
Amoxycillin	31 ± 8 ^b	49 ± 12	82 ± 3 ^a	75 ± 11
Cloxacillin	34 ± 8 ^b	17 ± 5 ^b	60 ± 13 ^b	43 ± 6 ^a
Nafcillin	42 ± 9 ^b	29 ± 10 ^b	94 ± 10	76 ± 6 ^b

Source: from Craven, Williams and Anderson (1982), by courtesy of *Comparative Immunology, Microbiology and Infectious Diseases*.

As Table 13.4, but mean of 4 (± SEM); see also Table 13.4 for footnotes.

The sensitivity of *S. aureus* to lysostaphin was also increased after cloxacillin or nafcillin exposure (Table 13.6). The increased sensitivity to muralytic enzymes may partially explain the greater killing effect of PMN. Extracts from leucocytes were more effective at killing penicillin-treated staphylococci than untreated bacteria (Efrati *et al.*, 1976), possibly as a result of muralytic enzymes present in the extracts, or indirectly through the facilitation of staphylococcal autolysins (Sela *et al.*, 1978). However, other workers consider that enhancement of bacterial killing is not dependent on a cooperative interaction with bacterial autolysins (Yourtee and Root, 1982) and the precise mechanism remains uncertain. From our results, it appears that the ability to enhance staphylococcal susceptibility to enzymic and cellular defence mechanisms is a feature of many (perhaps all) penicillins and interpretation of their relative value in this activity should be made with caution.

Since staphylococci surviving inside phagocytes are protected from the lethal action of penicillins, but may become sensitized to subsequent exposure to lysostaphin, it may be argued that they should also show an increased susceptibility to subsequent killing by fresh bovine PMN. However, we were unable to demonstrate this, possibly because of experimental limitations (Craven and Anderson, 1980b). Recent observations on enhanced susceptibility of penicillin pre-treated streptococci to killing by human PMN (Horne and Tomasz, 1981) indicated that this sensitization still occurred after growth of the streptococci with penicillin at pH 5.5 (conditions which offer protection against the lethal effects of the antibiotic). This implies that bacteria exposed

Table 13.6 SUSCEPTIBILITY TO KILLING BY 0.15 µg/ml LYSOSTAPHIN OF *Staphylococcus aureus* PRE-TREATED WITH PENICILLINS

	Oxford		M60	
	< MIC	> MIC	< MIC	> MIC
Amoxycillin	66 ± 26	92 ± 33	95 ± 10	95 ± 10
Cloxacillin	100 ± 29	33 ± 16 ^a	74 ± 9	52 ± 26
Nafcillin	35 ± 12 ^a	46 ± 17 ^a	50 ± 20	79 ± 38

Source: from Craven, Williams and Anderson (1982), by courtesy of *Comparative Immunology, Microbiology and Infectious Diseases*.

Values represent mean (± SEM) survival of penicillin-treated *S. aureus* as a percentage of control (untreated) survival.

Pre-treatment of *S. aureus*:

< MIC = growth for 18 h in presence of 1/4 MIC penicillins; mean of 4 (± SEM).

> MIC = brief (45 min) exposure to 10 × MIC penicillins; mean of 3 (± SEM).

From paired sample Student's 't' tests.

^a Significantly fewer survivors than in control, $P < 0.05$.

to penicillins while protected inside phagocytes by a moderately low intravacuolar pH may, in fact, become more susceptible to subsequent encounters with PMN. Bacteria surviving within those phagosomes which show maximum pH depression, perhaps to pH 5.0 (Reinitz, Paape and Mather, 1982) will be completely protected from both bactericidal and sensitization effects of penicillins (Craven and Anderson, 1982b).

Thus, the interaction of penicillins with bovine PMN in killing *S. aureus* may show either synergism or antagonism, according to the time of addition of penicillin, and may be modulated by the phagosomal pH. These interactions are summarized in Table 13.7.

The importance of these interactions *in vivo* was examined, using the mouse mastitis model. Endotoxin was injected into the glands 10 h before bacteria in order to draw PMN into the milk ducts, thus facilitating a rapid interaction immediately after bacterial inoculation. In these glands, significantly more cloxacillin- or nafcillin-treated *S. aureus* (at 1/4 MIC) were killed in the first hour than were untreated bacteria, but by 4 h no differences were found. No significant effects were seen with staphylococci pre-treated with 10 × MIC penicillins (Table 13.8). Thus, any synergistic effect of penicillin pre-treatment *in vivo* was short lived.

Table 13.7 SUGGESTED INFLUENCE OF PHAGOSOMAL pH ON KILLING OF *Staphylococcus aureus* BY PMN AND PENICILLINS

	pH 7.5→6.5	pH 6.5→5.5	pH less than 5.5
Survival of <i>S. aureus</i> within PMN	Low	Increasing	High
Action of penicillins on surviving <i>S. aureus</i>	Bactericidal (?)	Sub-lethal damage (tolerance)	No effect (complete protection)
Interaction of penicillins + PMN in killing <i>S. aureus</i>	Synergism	Indifference or antagonism	

Table 13.8 KILLING OF PENICILLIN-TREATED *Staphylococcus aureus* M60 IN ENDOTOXIN-STIMULATED MOUSE MAMMARY GLANDS

	Pre-treated at 1/4 MIC survival after:		Pre-treated at 10 × MIC survival after:	
	1 h	4 h	1 h	3 h
Control (untreated)	36.5	6.1	19.7	10.8
Cloxacillin (treated)	19.5	4.7	12.7	10.6
Nafcillin (treated)	14.5	7.3	12.0	11.1

Source: from Craven, Williams and Anderson (1982), by courtesy of *Comparative Immunology, Microbiology and Infectious Diseases*.

After treatment with penicillins, c. 10⁶ cfu M60 were inoculated into each mouse mammary gland 10 h after injection of 25 µg endotoxin.

Each value represents the geometric mean percentage survival of the initial bacterial inoculum and was obtained from total viable counts of 10 mammary glands (5 mice, 2 glands each).

In chronic mastitis in the mouse where, at the time of therapy, most bacteria were intracellular, neither nafcillin nor cloxacillin showed any therapeutic effect. In bovine mastitis, as in the mouse, a cooperative interaction of penicillins and PMN may occur in the initial encounter with staphylococci, but in the longer term it seems likely that protection of intracellular staphylococci from antibiotics will be the predominant feature.

Conclusion

Current attitudes towards control and treatment of bovine mastitis are divided between those who seek more effective antibiotic-based remedies and those who favour immunological approaches as preferred alternatives to antibiotic usage. It is increasingly apparent, however, that the outcome of antibiotic therapy is determined by the interactions of both antibiotics and immune mechanisms. Recognition of this fact may enable us to exploit favourable interactions and overcome adverse ones. Our preliminary work in this area has already revealed the significance of intracellular survival of staphylococci and consequent protection from antibiotic action. The use of suitable combinations of antibiotics which may kill both intracellular and free bacteria within the mammary gland may represent a first step forward. Further investigation of the influence of intraphagosomal pH on survival and protection of bacteria may suggest new strategies. The somewhat empirical combinations of antibiotics and anti-inflammatory drugs in use today may need re-evaluation as our knowledge of interactions of antibiotics and host defence mechanisms increases.

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CHEMOPROPHYLAXIS IN BOVINE MASTITIS

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Introduction

Bovine mastitis is an inflammation of the bovine mammary gland due to infectious (septic) or non-infectious (aseptic) causes, although the latter appear to have little economic significance.

A wide spectrum of microorganisms has been isolated from milk and mammary tissue, but over 80% of infections are associated with Gram-positive cocci. In the UK, the earlier predominance of streptococcal infections (*Streptococcus agalactiae*, *S. dysgalactiae* and *S. uberis*) appears to have been lost and currently *Staphylococcus aureus* is involved in the majority of infections. In recent years *Escherichia coli* has received more attention as a mastitis pathogen. *Corynebacterium pyogenes* is involved in the acute disease known as summer mastitis which affects dry cows and heifers not only in midsummer, but in midwinter too. *C. bovis* and micrococci which normally produce little inflammation may be present, but are usually ignored as potential pathogens. The importance of other infections such as *Leptospira* and *Mycoplasma* spp. is uncertain. Milking machines are commonly considered to be a major factor influencing the prevalence of mastitis and, although this may well be true, new mastitis infections can occur in un milked cows, e.g. heifers before calving and dry cows.

There is an association between the total cell count of milk and the presence of a pathogen. Quarters of the udder secreting milk with more than 500 000 cells/ml are classified as mastitic (Wilson and Richards, 1980). Regular herd milk cell counts have been available to all dairy farmers in England and Wales from the Milk Marketing Board since 1977.

Septic mastitis occurs in all dairy herds and inflicts economic losses through reduced milk yield, cost of treatment, wastage of milk contaminated by antibiotics and discarded, and death or culling of badly affected cattle. The disease may be clinical, i.e. detected by clinical examination of the cow or her milk, or sub-clinical, i.e. detectable only by additional diagnostic tests.

Financial losses for individual dairy farmers may be considerable. The loss from clinical mastitis in a 100-cow herd has been estimated at £800–£1000 and the additional loss from sub-clinical disease at £2000–£2500 p.a. (Hibbit, 1983). The annual national loss in the UK has been estimated at £87 million or approximately £27 for each cow in the country (Booth, 1981).

Some bacteria may live in the udder without promoting significant changes

in tissues or secretions. Others are capable of flourishing to such an extent that they both ferment the milk and destroy the tissues. They may also produce toxins which may be absorbed from the udder and produce serious systemic effects.

The great majority of infections are believed to enter the udder through the teat canal both during lactation and during the dry period (Schultze and Paape, 1981). In order to establish themselves in the udder the microorganisms have to overcome specific and non-specific mammary gland defence mechanisms which act in unison against incoming infection. The result of this encounter varies from clearance of the infection to death of the animal. Most commonly an intermediate state of affairs prevails, the animal being unable to clear the infection from the udder, but appearing quite healthy on clinical examination. In such sub-clinical infections, the causal organisms are held in check, but at the cost of a reduction in milk yield. The condition may flare up into clinical mastitis at any time.

When the stockman sees signs of clinical mastitis, antibiotic therapy is usually given. In response to this treatment, clinical signs of mastitis usually subside rapidly and often (approximately 63% of cases) infection is cleared, but not always (Griffin, Dodd and Bramley, 1982). Unfortunately, antibiotic residues in milk from such cattle may inhibit the fermentation processes involved in cheese and yoghurt production and allergies to residues of penicillin and other antibiotics are important in public health. Control measures which reduce or avoid such residues in milk are therefore to be preferred.

Traditionally, control of mastitis in the dairy herd has been in the hands of the dairy farmer and his veterinary surgeon, with investigational and advisory support from the Veterinary Investigation and Dairy Husbandry Advisory Services of the Ministry of Agriculture, Fisheries and Food (MAFF). Other parties, such as the Milk Marketing Board (MMB), the National Institute for Research in Dairying (NIRD) and the Central Veterinary Laboratory, Weybridge, UK (CVL), have maintained an interest.

In 1972, a formal National Mastitis Awareness Scheme which brought the various interested parties together was launched. The basic mastitis control measures advocated were those developed earlier by the NIRD and CVL (Dodd and Neave, 1970). These consisted essentially of a five-point programme involving milking-machine testing, teat dipping, dry cow therapy, correct clinical treatment and records of such treatment, and culling chronic cases of mastitis.

By 1977 (Wilson and Richards, 1980), 76% of herds of 100 cows or more were using teat dipping and dry cow therapy, but a somewhat lower percentage of smaller herds adopted these procedures (*Table 14.1*). The authors associated a fall from 50% (*Figure 14.1*) of cows infected with a major pathogen in 1967 to 32% in 1977 (*Table 14.2*) with the use of these control measures.

Many of the elements of the NIRD-CVL programme were well known at the time of its inception; the novelty lay in their combination in a pragmatic programme of proven effect in reducing both the number of new udder infections and the number (and persistence) of established infections. Nevertheless, the individual components were not necessarily fully evaluated, in the sense that the individual contributions of each could not easily be separated from the effect of other changes.

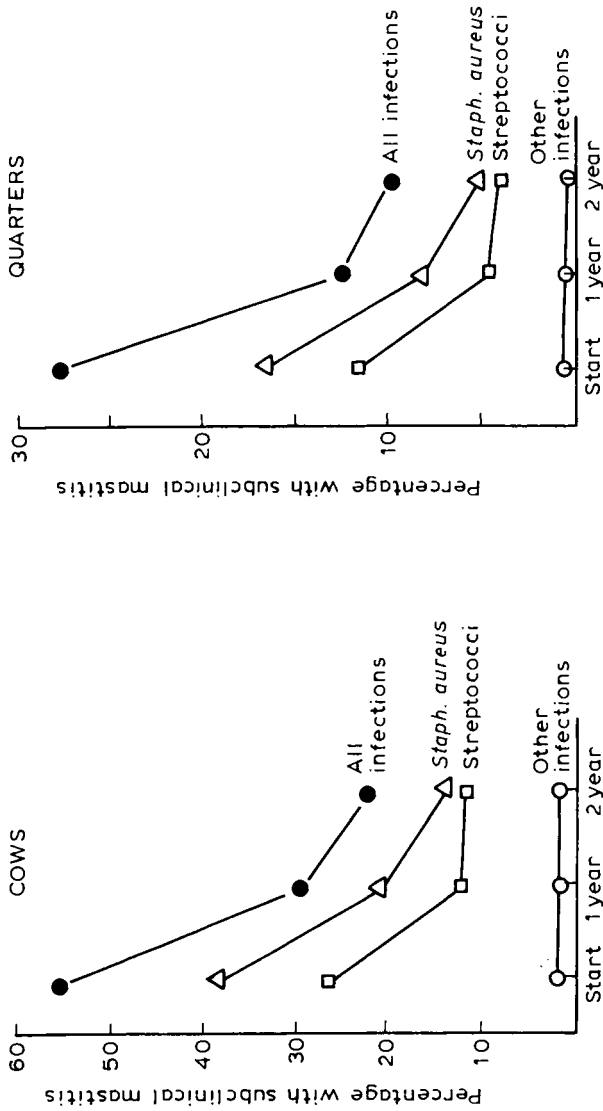


Figure 14.1 The reduction in the proportion of cows and quarters with sub-clinical mastitis in 30 herds in the first 2 years after the introduction of a simple control system (MF3) (from Dodd and Neave, 1970, by courtesy of the National Institute for Research in Dairying)

Table 14.1 UK MASTITIS SURVEY—HERDS EXAMINED BY HERD SIZE GROUP

	<i>Herd size group</i>					
	10-20	21-30	31-45	46-60	61-100	100+
Number of herds examined	27	49	88	89	155	93
Number of herds in census	8372	8339	11 245	8617	11 415	5456
Sampling fraction (%)	0.32	0.59	0.78	1.03	1.36	1.70
Proportion of quarters infected with a major pathogen (%)	21.2	20.7	16.7	15.6	14.5	11.9
Proportion of herds using teat dipping and dry cow therapy (%)	26.9	44.9	60.9	59.1	59.4	76.3

Source: from Wilson and Richards (1980), by courtesy of *Veterinary Record*.

Table 14.2 UK MASTITIS SURVEY—PREVALENCE OF INFECTION, BY PATHOGEN

<i>Pathogen</i>	<i>Infected herds</i>		<i>Infected cows</i>		<i>Infected quarters</i>	
	(no.)	(%)	(no.)	(%)	(no.)	(%)
<i>Streptococcus agalactiae</i>	197	39.3	1 992	7.2	3 694	3.4
<i>S. dysgalactiae</i>	311	62.1	968	3.5	1 195	1.1
<i>S. uberis</i>	369	73.7	1 267	4.6	1 610	1.5
<i>Staphylococcus pyogenes</i>	467	93.2	5 689	20.7	8 790	8.1
Other major pathogens	261	52.1	624	2.3	796	0.7
Any major pathogen	500	99.8	8 807	32.0	15 382	14.1
<i>Corynebacterium bovis</i> , micrococci	501	100.0	19 279	70.0	47 523	43.5
Total sampled	501	100.0	27 526	100.0	109 160	100.0

Source: from Wilson and Richards (1980), by courtesy of *Veterinary Record*.

The use of these or any control measures has to be viewed in the context of a National Herd, which has adapted to severe economic pressures by substantial changes in structure and management. For example, since 1969 (Anon, 1982) the total number of dairy cows in England and Wales remained static at about 2.7 million, while the number of dairy farms has fallen substantially. In compensation for this decline, average herd size has increased from 33 to 63. The average milk yield per cow has risen from 3775 to 4785 l. over the same period. M. Slater (1983, personal communication), in discussing modern intensive dairy farming, has described the gross margin target of £1070/ha on 170 ha for farmers in the upper quartile. The need for this economic productivity leads to the situation encountered where one man tends 24 cows simultaneously in a herring-bone parlour and has on average only 3 min daily to devote to each cow. To be integrated into such a routine, control methods must be simple, easily and rapidly applied and avoid the need for detailed investigation or diagnostic procedures for individual cows. The fact that larger herds tend to adopt the five-point programme most readily suggests that these criteria of acceptability have been met.

Adoption of a practical control programme does not inhibit investigation or introduction of alternative or supplementary means of control, and work

continues on improved husbandry, milking-machine design, hygiene and maintenance, selection for inherited resistance to mastitis and induction of passive or active immunity. So far, however, experimental vaccines have proved inadequate (Opdebeeck, 1982). There is a need for further study of routes of immunization, timing in relation to age and stage of lactation, vaccine components, antigenicity of organisms, optimum concentration of antigens and most efficient adjuvants.

The five-point programme depends heavily, but by no means exclusively, on chemoprophylaxis or the strategic administration to dairy cattle of compounds of specific activity in order to control bovine mastitis. Immediate objectives of such chemoprophylaxis may include enhancement of mammary gland defence mechanisms, e.g. immunomodulation by levamisole, reduction of established mammary infections, e.g. dry cow therapy, and limitation of teat exposure to potential pathogens, e.g. teat dipping.

Immunomodulation by levamisole

Tetramisole is 2,3,5,6-tetrahydro-6-phenylimidazol (2,1-b) thiazole whose anthelmintic properties were first reported by Thienpont and his co-workers (1966) and later discovered to be predominantly due to the l-isomer, levamisole.

Renoux and Renoux (1973) reported improved protection against challenge infection by *Brucella abortus* in vaccinated mice treated with tetramisole. This led to an avalanche of reports of the immunomodulating effects of levamisole and its potential benefits in restoring host defence mechanisms.

Immunotherapy is basically the treatment of a host compromised by a defective immune response, and has a potential role in both treatment and prophylaxis when restoration of immune function is required. Levamisole's modulating effects on the immune system can be summarized as follows (Brugmans, 1978): induced maturation of T cells in immunologically immature animals and restoration of T lymphocyte, neutrophil and phagocyte function in compromised hosts. It has no direct effect on B lymphocytes. At therapeutic dosage, levamisole does not stimulate immune responses above normal physiological level.

In treatments designed to produce immunomodulation in man, levamisole has generally been administered intermittently on 2, 3 or 4 consecutive days every week, every fortnight or at each clinical episode. Once immune functions have been restored they cannot be stimulated to a greater pitch by levamisole, nor is restoration necessarily permanent. When levamisole therapy is withdrawn, relapse may ensue. Levamisole immunotherapy should be considered as an aid to treatment and not as a permanent cure for a basic immunodeficiency.

The extensive literature regarding clinical effects in man and experimental data derived from *in vivo* and *in vitro* studies has been reviewed elsewhere (Symoens and Rosenthal, 1977; Symoens, 1977; Brugmans, 1978).

Clinical data relating to immunodulation by levamisole in domesticated animals is relatively sparse, but is nevertheless sufficient to demonstrate that beneficial effects are associated with such immunotherapy in pigs (Misley and Sarkozy, 1979); Reyero, Stockl and Thalhammer, 1979; Best, 1980), poultry

(Kulkarni *et al.*, 1973; Panigraphy, *et al.*, 1979; Soppi *et al.*, 1979); dogs (Wilkinson, 1979; Lowe, 1979; D.B. Harker, 1981, personal communication), and sheep (Hogarth-Scott, Liardet and Morris, 1980; Mitchell and Armour, 1981).

In cattle, repeated doses of levamisole at weekly intervals during the last 2 months of pregnancy have been shown to reduce the incidence and severity of mastitis and, through an effect on their colostrum, to enhance prospects of survival for their offspring.

Preliminary studies (Ovadia, Flesh and Nelken, 1978) involved 15 pregnant cows treated parenterally with 15 ml of 10% levamisole solution (1500 mg levamisole) weekly for 4 consecutive weeks during their dry period. A control group consisted of 16 untreated pregnant cows from the same herd. The frequency of mastitis in these cattle is shown in *Table 14.3*.

Lymphocyte transformation tests on the two groups of cows showed that the phytohaemagglutinin index was, in general, higher for cows treated with levamisole. Both the incidence and severity of clinical mastitis were reduced in the groups of cows pre-treated with levamisole.

Studies in Japan (Ishikawa *et al.*, 1982) showed that in normal quarter milk the relative amount of immunoglobulin increased in 20 cows treated orally with 7.5 mg/kg levamisole hydrochloride. Severity of mastitis was reduced as assessed by scores in a modified California Mastitis Test on quarter fore milk samples. The authors concluded that levamisole is of value in the treatment of sub-clinical bovine mastitis.

Results of more extensive work (Flesh, Harel and Nelken, 1982), involving parenteral administration of 10 mg/kg levamisole to 1204 pregnant cattle every week for 6 consecutive weeks before calving, are shown in *Table 14.4*.

Table 14.3 FREQUENCY OF MASTITIS AND BACTERIOLOGICAL FINDINGS IN LEVAMISOLE-TREATED AND CONTROL COWS

Group	Total number of cows	No. with clinical mastitis	No. with positive bacteriology		
			<i>Staphylococcus aureus</i>	Coliform	Other
Levamisole-treated	15	1	4	1	0
Control	16	5	7	1	1

Source: from Flesh, Harel and Nelken (1982), by courtesy of *Veterinary Record*.

Table 14.4 EFFECT OF LEVAMISOLE ON MASTITIS

	No. of cows	Acute mastitis (clinical)	Percentage
Control	3105	298	9.6
treatment groups	1204	45	3.7
Primipara control groups	300	39	13.0
Primipara treatment groups	165	1	6.0

Source: from Flesh, Harel and Nelken (1982), by courtesy of *Veterinary Record*.

Levamisole alone had no effect in animals treated for acute mastitis, i.e. the effect was prophylactic not therapeutic.

It is concluded that levamisole, when administered at weekly intervals on 4–6 occasions to cows in their dry period, aids the reduction of clinical mastitis during the ensuing lactation and may thus also reduce the need for antibiotic treatment of lactating cattle.

Dry cow therapy

Control measures against bovine mastitis cannot be limited to lactation alone, as cattle may enter the dry period with established sub-clinical infection and they are susceptible to new infection during the dry period. This susceptibility varies (Hoare *et al.*, 1977) and is most pronounced within 10 days of drying off and again within 10 days before calving. In order to achieve persistent antibacterial activity within the udder, over the dry period, intramammary administration of an effective slow-release antibacterial formulation is usually advocated. However, dry cow therapy has the advantages that diagnosis is not required, persistent infections are treated annually, more time for regeneration of secretory tissue is available than during treatment in lactation and antibiotic use during lactation (and attendant problems) is reduced.

B. Templemore-Finlayson (1978, personal communication) suggested that slow-release properties may not be essential if the periods of major susceptibility were covered. He investigated the efficacy of a rapid release formulation of penicillin G and dihydrostreptomycin sulphate during an outbreak of mastitis in a dairy herd, where post-milking teat dipping was used as the sole means of controlling mastitis. A sub-clinical infection of *Stap. aureus* and *S. agalactiae* was confirmed in a group of cows at the end of lactation. Ten animals were allocated to intramammary treatment at drying off and again 10 days before the expected date of calving. In the treatment group, 25 out of 33 (75%) infected quarters were cleared at calving, while only 8 out of 18 (44%) were cleared in controls. The author concluded that a degree of self-cure occurred in the dry period, but that this was significantly improved by the use of the dry cow therapy. Antibacterial activity was absent from the milk 2 days after calving.

Dry cow therapy may increase the likelihood of antibiotic-resistant strains arising and these may have significance for public health. It may also be associated with an apparently increased incidence of Gram-negative infections (see Teat Dipping below).

Teat dipping

The occurrence of new mammary infection during lactation can be reduced by reduced exposure of the teat to pathogenic organisms. Several measures may aid reduced exposure, e.g. clean housing conditions, isolation of infected animals, antibiotic therapy, cleaning the milking machine, treating the milking cluster to reduce transfer of infection from cow to cow and application of antiseptic to the teats before and after milking. In fact, the application of antiseptic solutions (usually iodophor, chlorhexidine or sodium hypochlorite)

Table 14.5 ISOLATION OF PATHOGENS FROM CLINICAL MASTITIS, 1963-80

Year	No. of herds	Percentage of clinical samples containing:				
		<i>Staphylococcus aureus</i>	<i>Streptococcus agalactiae</i>	<i>Streptococcus dysgalactiae</i>	<i>Streptococcus uberis</i>	<i>Escherichia coli</i>
1963	14	35	2	33	8	3
1966-70	30	33	2	18	21	7
1980	378	15	2	7	11	20

Source: from Bramley (1981), by courtesy of the BCVA.

Table 14.6 PERSISTENCE OF ANTIBACTERIAL ACTIVITY ON TEAT SKIN TREATED WITH F.2402 AGAINST *Staphylococcus aureus*

Cow no.	Log. 10 <i>Staphylococcus aureus</i> recovered from teats treated with F.2402 and placebo when contaminated (hours after drying)			
	4½ h		17 h	
	F.2402	Placebo	F.2402	Placebo
372	<2.18 (LH) 3.29 (RF)	2.66 (LF) 5.12 (RH)	5.33 (LH) 5.71 (RF)	5.74 (LF) 5.72 (RH)
6829	4.06 (LF) <2.18 (RH)	5.15 (LH) 5.66 (RF)	4.22 (LF) 4.46 (RH)	5.69 (LH) 5.91 (RF)
16	2.66 (LH) 2.78 (RF)	4.74 (LF) 5.23 (RH)	4.70 (LH) 5.66 (RF)	4.78 (LF) 5.54 (RH)
981	3.48 (LF) 3.13 (RH)	3.78 (LH) 5.30 (RF)	4.52 (LF) 4.85 (RH)	3.99 (LH) 5.26 (RF)
Mean	2.97	4.70	4.93	5.33
Difference		1.73		0.4
S.E.		0.43		0.27
Mean percentage reduction and 95% confidence limits		98% (84.6-99.8%)		60% (0-90%)

Source: from Forse *et al.* (1970), by courtesy of *Veterinary Record*.

to teat skin after milking is a widely accepted practice in the UK dairy industry. These materials are all highly bactericidal on teat skin and help to ensure that pathogenic bacteria on the teat immediately after milking are destroyed, irrespective of their source. Colonization may thus be prevented and existing colonies removed from the teat orifice. Infection of teat sores by microorganisms is also prevented or removed, thereby reducing an important source of infection and aiding the healing of the lesion.

The originators of the NIRD-CVL mastitis control programme forecast that, as the effect of the programme was felt and sub-clinical infection due to staphylococci and streptococci declined, other relatively uncommon infections would become of relatively greater importance. This appears to be true, both on a national scale (Wilson and Richards, 1980; Bramley, 1981) and in individual problem herds (Table 14.5), in that an apparent, if not an actual, increase in so-called environmental mastitis (due to *S. uberis* and *E. coli*) has been observed. Robinson, Jackson and Marr (1983), as a result of observations in veterinary practice, believe that the increase in some herds is real and associated with the use of teat dipping and dry cow therapy.

Forse *et al.* (1970) first demonstrated the persistence of chlorhexidine's antibacterial activity on cows teats (Table 14.6) and suggested that this may be an important aspect of control of infection by teat dipping. More recently (Godinho and Bramley, 1980), the persistence of antibacterial activity on teat skin was tested for ethanol, iodophor and chlorhexidine using *S. uberis* and *E. coli* as test organisms either 15 min or 15 h after teat dipping (Table 14.7).

Table 14.7 EFFECT OF ONCE DAILY ETHANOL, IODOPHOR OR CHLORHEXIDINE TEAT DISINFECTION ON NEW UDDER INFECTION IN UNMILKED COWS

Pathogen(s) isolated	No. quarters becoming infected			
	No dip	Ethanol	Iodophor	Chlorhexidine
<i>Streptococcus uberis</i>	23	19	20	12
<i>Escherichia coli</i>	2	1	1	2
<i>S. uberis</i> and <i>E. coli</i>	0	1	0	0
Clinical (no bacteria isolated)	1	5	1	1
Total ^a	26	26	22	15

Source: from Godinho and Bramley (1980), by courtesy of *British Veterinary Journal*.

^a Totals joined by the same horizontal line do not differ significantly ($P < 0.05$).

Fewer quarters became infected following the use of chlorhexidine than with any other treatment. Currently, a chlorhexidine teat dip giving prolonged protection after teat dipping is being evaluated by NIRD on two commercial farms (Leitch, 1983). Half the animals are being teat dipped with an iodophor and the other half with chlorhexidine. New infections are being assessed over a period of 6 months. So far, results against environmental mastitis are promising.

To be effective and acceptable for teat dipping, an antiseptic preparation should be active against all types of bacteria capable of causing udder infections, rapidly bactericidal in the presence of milk and on teat skin, non-irritant on prolonged use, with persistent antibacterial activity between milkings and free of residues in the milk.

Desirable though they may be, none of these properties can easily be appreciated by the user, who tends to base his selection on the cosmetic effect of teat dipping on teat skin, i.e. production of smooth, supple teat skin and a marked reduction in skin lesions, particularly chaps and sunburn. This is by no means an irrelevant judgement since both chaps, i.e. fissures of the skin due to loss of elasticity, and sunburn can lead to pain and difficulty at milking time and chaps have been associated with new teat infections (Figure 14.2). The emollient properties of teat dips are therefore extremely important, especially to the purchaser.

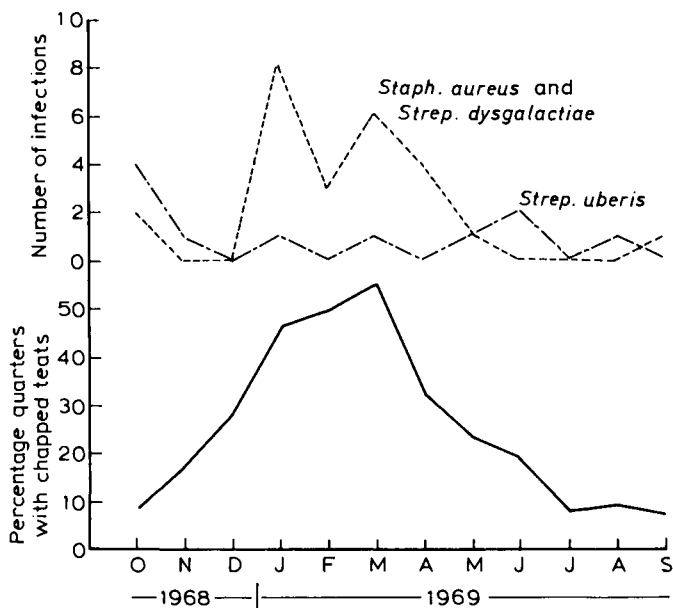


Figure 14.2 The relationship between teat chapping and new infection—note the rise in *Staphylococcus aureus* and *Streptococcus dysgalactiae* infections at the time when chapped teats are most common, and the lack of relationship with *S. uberis* infection. Data from 1 herd in MFE 3(2) during a 12-month period (from Dodd and Neave, 1970, by courtesy of the National Institute for Research in Dairying)

Sunburn affects unpigmented skin and is more common on the front teats, whereas chapping is most common on the hind teats (Jackson, 1972), although A. Richardson (personal communication) found in a study of 19 herds that the incidence was seasonal, occurring in May and June, with front and hind teats equally affected. He postulated that addition of emollients to teat dips, although usually beneficial, may occasionally make matters worse, e.g. under very dry, cold conditions when hygroscopic emollients such as glycerine remove water from the epidermis and thus aggravate the condition they were used to prevent.

This chapter indicates that antibacterial activity is not the only important element in chemoprophylaxis and that the fight against mastitis does not depend on chemoprophylaxis alone. In order to succeed, a marriage between medicine and many other factors is essential.

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RECENT ADVANCES IN THE USE OF β -LACTAM ANTIBIOTICS FOR THE TREATMENT OF MASTITIS

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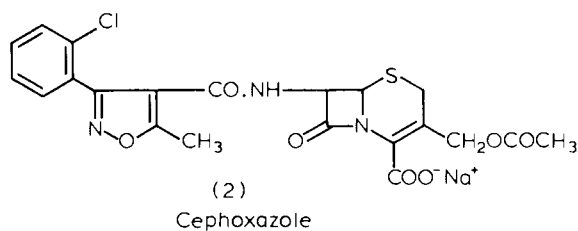
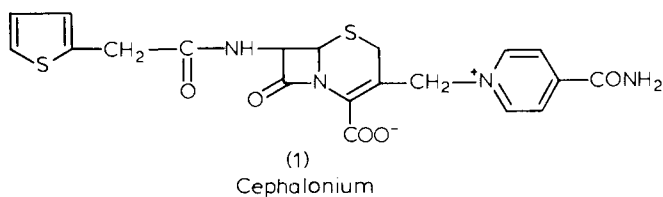
Mastitis is inflammation of the udder usually caused by bacterial infection. Recently, it was reported that about £100 million a year is lost by the British dairy industry because of the disease (*The Times*, 24 January 1983). According to the Central Veterinary Laboratory, in the 12 months to June 1982 there had been 8773 cases of mastitis from 25 800 cows in the 220 herds included in their surveillance scheme; this proportion, reflected in the total dairy cow population of 3.25 million in the UK, represents over 1 million cases a year. Losses due to mastitis are caused by the need to discard mastitic milk and milk containing antibiotic following treatment, loss of yield in infected cows, the need to cull cows with severe infection, and the cost of treatment. Cows developing clinical mastitis during lactation are usually treated by infusing antibiotic into the teat canal. It is also customary to give prophylactic antibiotic treatment during the dry period.

Cephalosporins and other β -lactam antibiotics used for intramammary therapy

At the last international symposium on antibiotics in agriculture, Harris and Muggleton (1977) reported an increase in the number of penicillin-resistant strains of staphylococci associated with mastitis, and Boulton and Ross (1977) stated that 70% of bovine staphylococci produced penicillinases and thus showed resistance to penicillin. The fact that cephalosporins are resistant to virtually all staphylococcal penicillinases stimulated interest in preparations based on semi-synthetic cephalosporins for the control of bovine mastitis. When inactivated by β -lactamases, penicillins degrade to leave a moiety capable of producing an allergic reaction in individuals sensitive to penicillin, whereas the cephalosporins used for mastitis control break down to products not likely to cause an allergic response if present in milk.

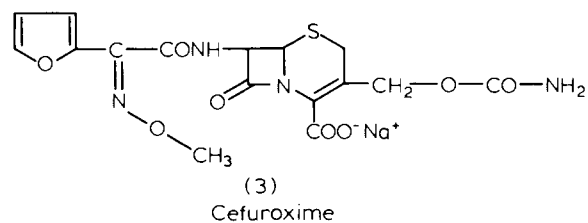
Two intramammary preparations were developed. One contains the relatively insoluble cephalonium (1) for use in dry cows where the physical properties of the antibiotic and formulation base ensure prolonged antibiotic activity in the dry udder. The other preparation contains cephoxazole (2).

When used in combination with penicillin, cephoxazole may inactivate β -lactamases produced by Gram-negative organisms and thus protect penicillin (Boulton and Ross, 1977), an effect similar to that produced by clavulanic



acid. The combination is not quite as effective *in vivo* as expected, however, due to pharmacokinetic differences between the cephalosporin and penicillin, a difficulty that occurs with any combination of this kind.

Cefuroxime (3) combines, in one antibiotic, resistance to β -lactamases



produced by both Gram-positive and Gram-negative strains, a wide spectrum of activity and good pharmacokinetic properties (O'Callaghan, 1977).

Evaluation of cefuroxime for mastitis control

In a recent study (R. Cross and A.M. Davies, unpublished), cefuroxime was evaluated for the control of mastitis in milking cows. The results provided information on the potential of the antibiotic for mastitis control, and included a survey of pathogens associated with mastitis in the UK during 1982 and 1983.

PRELIMINARY STUDIES

The minimum inhibitory concentration (MIC) of cefuroxime was determined against a range of bacterial strains including mastitis pathogens. Most Gram-positive and Gram-negative strains were sensitive to low levels of cefuroxime when examined by conventional methods and when tested in the presence of whole milk.

A preparation of cefuroxime in a quick-release base was infused into healthy cows. Pre- and post-infusion milk samples were taken from these animals and cell counts were determined using a Coulter milk cell counter. The results are shown in *Figure 15.1*. Cell counts of <500 000/ml were considered normal, and a cell count of >1.0 million/ml in healthy cows indicated irritancy. Irritant preparations tend to give a sharp rise in cell count following the first infusion, the counts usually returning to normal some time after the last infusion. It was quite clear from the results shown that the cefuroxime was non-irritant as there was no rise in cell count after treatment, and the counts showed only normal fluctuations.

Post-infusion samples were assayed by the agar diffusion technique to determine the cefuroxime concentrations, and the results are shown in *Figure 15.2*. Three treatments at consecutive milkings maintained levels of antibiotic in the milk that exceeded the geometric mean MIC value of both Gram-positive and Gram-negative organisms for at least 5 milkings. Twenty-four-hourly treatments extended the period that the effective level of antibiotic was maintained in the milk.

Results of a very sensitive test which detects residual concentrations of antibiotic in milk, and which was based on the inhibition of *Bacillus stearothermophilus* var. *calidolactis*, indicated that milk from treated animals was free from cefuroxime residues 60 h (5 milkings) after the last infusion. Milk from animals given cefuroxime therapy should therefore be discarded during treatment and for 48 h (4 milkings) after the last infusion. The total volume of milk discarded in commercial practice would be greater with the 24-hourly-treatment regime (8 milkings instead of 6 milkings), and the difference may be seen as the price of maintaining a therapeutic level of antibiotic in the udder for a longer period.

These results showed that cefuroxime was potentially a suitable antibiotic for the treatment of mastitis, but more evidence was required to confirm the activity against the range of species currently causing mastitis in the UK, and therefore a field study was organized. Practising veterinarians were asked to select cows with clinical mastitis, and to take a pre-infusion milk sample from each infected quarter. The samples were immediately sent by first-class post to the investigating laboratory for bacteriological examination.

On receipt, the samples were plated neat and as a 1:1000 dilution in sterile pH 7.0 phosphate buffer onto three media. Streptococci were isolated on Oxoid Edwards Crystal Violet medium with 5% horse blood added, and Gram-negative bacteria were isolated on Oxoid Eosin Methylene Blue agar. In addition, samples were plated on Oxoid Blood agar base with 7.5% horse blood added. All plates were incubated for 48 h at 37°C before examination. All streptococci and Gram-negative organisms were isolated and identified using standard laboratory techniques based on the *Appareils et Procédés*

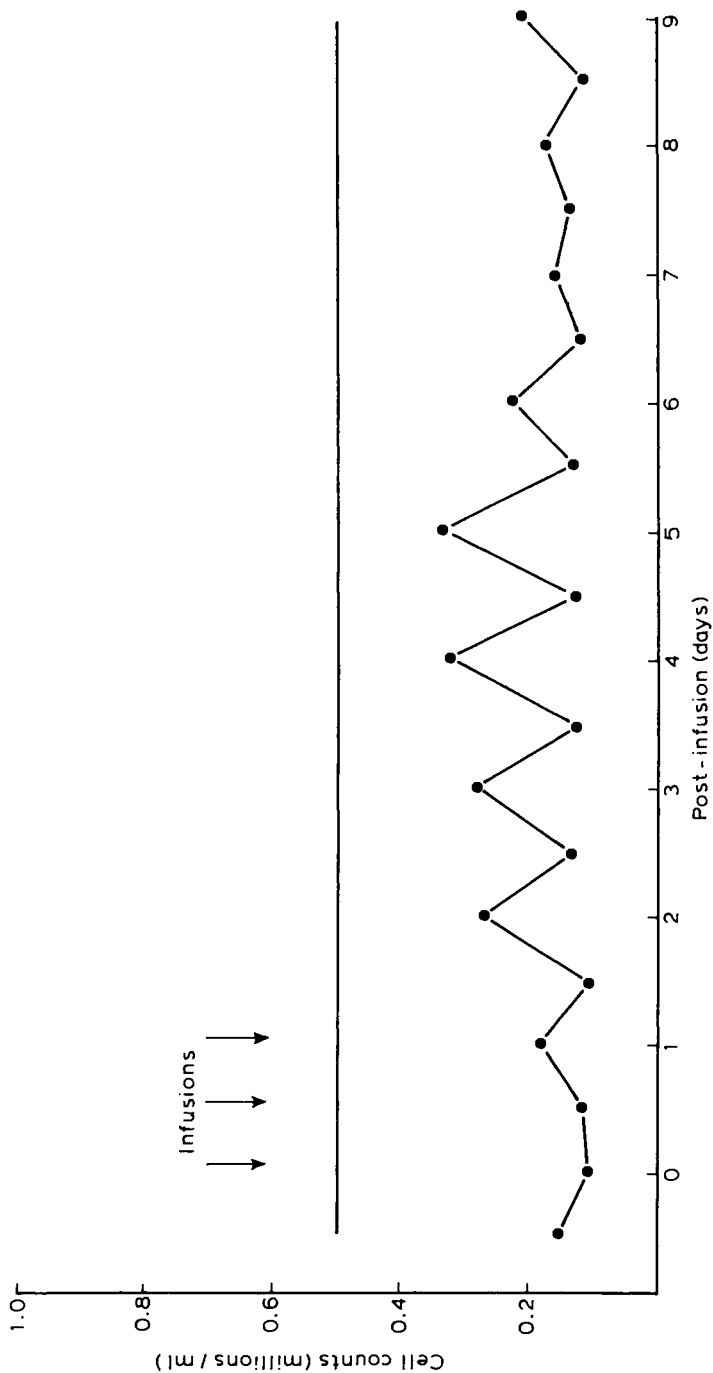


Figure 15.1 The acceptability of 250 mg cefuroxime

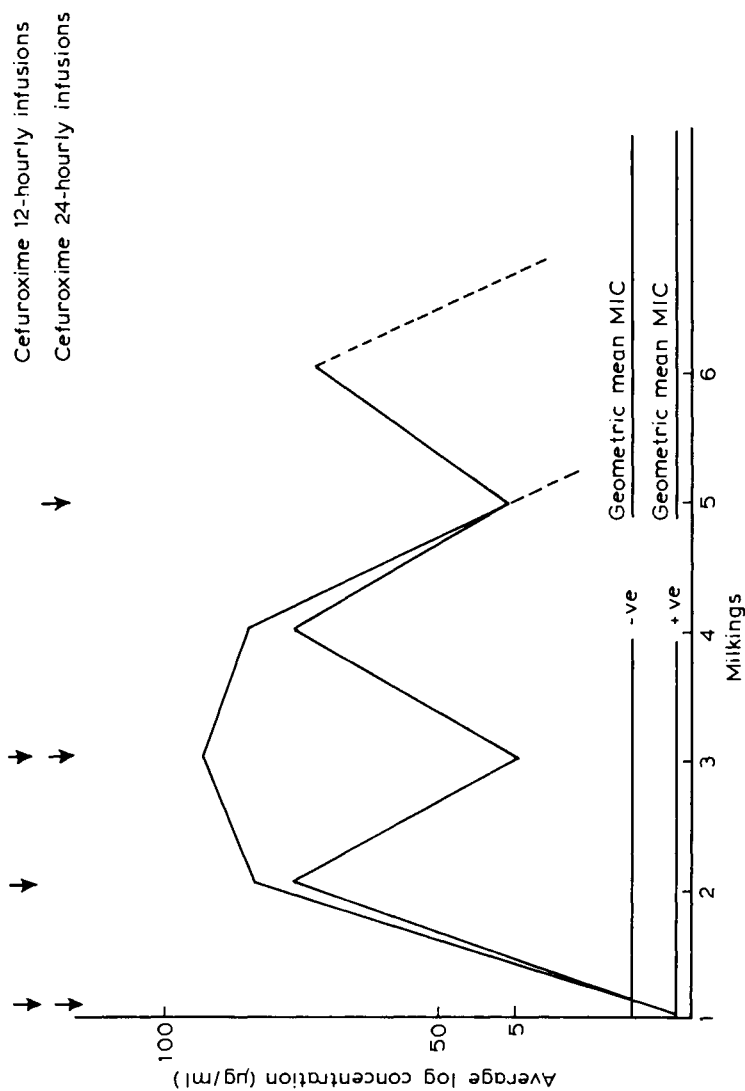


Figure 15.2 Excretion of 250 mg cefuroxime (-ve, Gram-negative; +ve, Gram-positive)

d'Identification (API) system. All other strains were cloned and identified using methods described by Cowan (1974).

There were 439 bacterial strains isolated. The distribution of strains within the major groups of pathogens (*Figure 15.3*) probably reflects the composition of the pathogen population currently causing bovine mastitis in the UK. More than half the strains isolated were Gram-positive (64%), and the largest groups comprised *Streptococcus uberis* (25%), *Escherichia coli* (19%) and *Staphylococcus aureus* (12%). There was an unexpectedly high percentage of *Serratia* strains and a possible explanation for this will be discussed later.

All 439 isolates were tested for sensitivity. Inocula were prepared by growing the strains in Tryptic Soya broth (supplemented with 5% horse serum for fastidious strains) overnight at 37°C. After incubation, the inoculum density was standardized by dilution with 0.15 mol/l pH 7.0 phosphate buffer. Most broth cultures required 1:100 dilution, but streptococci were usually diluted 1:10, and corynebacteria were plated neat. Mueller Hinton agar plates (supplemented with 5% horse blood for fastidious organisms) were dried at 37°C for 15–30 min, then inoculated with a sterile cotton wool swab dipped into the diluted inoculum and streaked across the surface of the agar. Antibiotic sensitivity discs were placed on the agar surface using an 'Oxoid' dispenser. After the plates were incubated overnight at 37°C, zones of inhibition were measured and recorded to the nearest 0.1 mm using automated calipers interfaced with a Hewlett Packard HP85 computer, which was programmed to supply a print-out of zone diameter and the sensitivity based on the manufacturer's recommended critical zone sizes. Commercially available sensitivity discs of cefuroxime, penicillin, streptomycin, Streptopen (Glaxo) (streptomycin and penicillin) and Cepoxillin (Glaxo) (cephoxazole and penicillin) were used and the strains were categorized as sensitive, intermediate or resistant to each antibiotic or antibiotic combination.

The number of strains tested, and the number of strains sensitive to each antibiotic or antibiotic combination, are shown in *Table 15.1*. The broad spectrum of activity of cefuroxime was demonstrated by the fact that 85% of all strains tested were sensitive, with no marked difference between Gram-positive and Gram-negative strains. *Serratia* strains, however, were relatively resistant to all the antibiotics and antibiotic combinations tested. As expected, penicillin was not particularly active against Gram-negative strains, and streptomycin failed to inhibit many Gram-positive organisms. The combination of the aminoglycoside and the β -lactam antibiotic in Streptopen showed the same broad-spectrum activity as previously reported by Evans *et al.* (1975). The combination inhibited 83% of the strains tested, compared with 57% inhibited by each antibiotic when used separately. The resistance of cefuroxime to staphylococcal penicillinase is evidenced by comparison of the number of strains of staphylococci sensitive to penicillin (51%) with the number sensitive to cefuroxime (99%).

Nitrocefin, the semi-synthetic cephalosporin which is extraordinarily susceptible to attack by β -lactamases from all sources, and forms a deep cherry-red compound when hydrolysed (O'Callaghan *et al.*, 1972) was used to confirm the production of β -lactamase from penicillin-resistant staphylococci which were sensitive to cefuroxime. All the *Escherichia coli* strains were resistant to penicillin, but 14 were sensitive to a combination of penicillin and cephexazole (Cepoxillin), thus indicating the protective effect of cephexazole

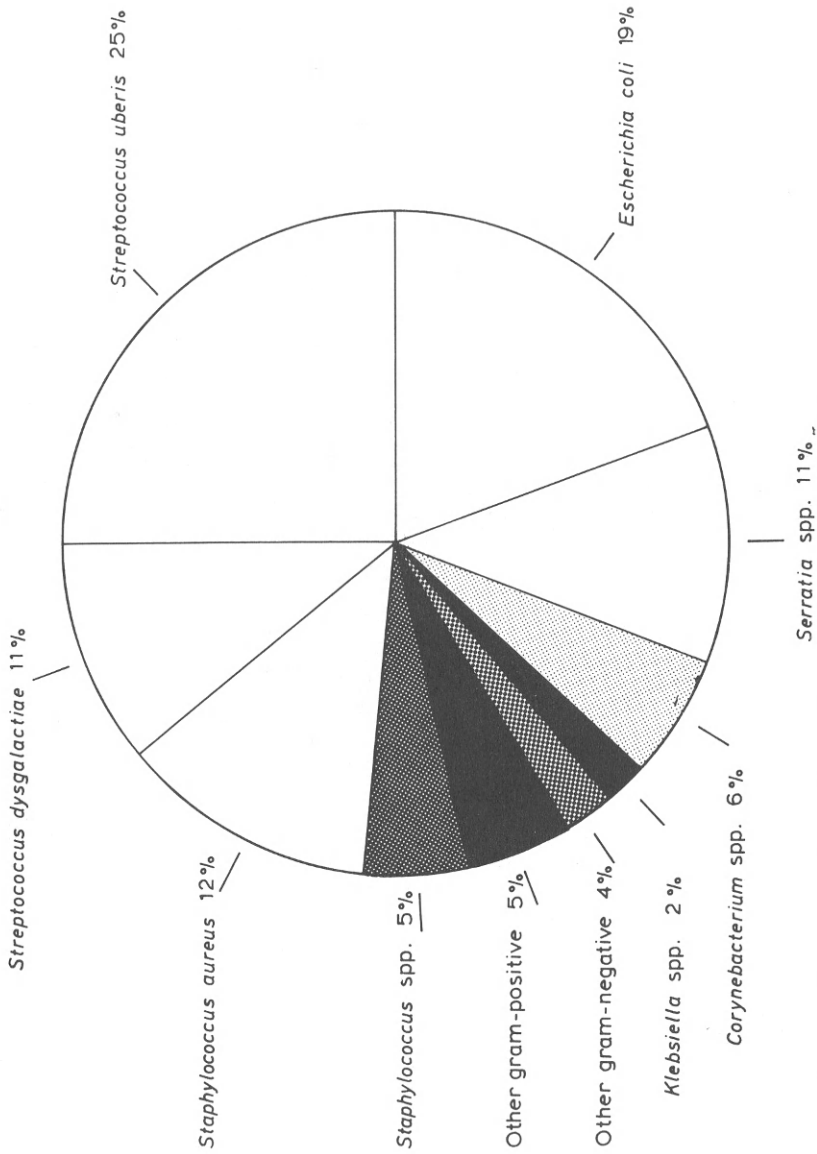


Figure 15.3 Pathogens isolated from 400 cases of bovine mastitis (1982-83)

Table 15.1 THE SENSITIVITY OF STRAINS ISOLATED FROM 400 CASES OF BOVINE MASTITIS (1982-83)

Strain	Total	Percentage	CXM	P	Number sensitive ^a		SXP	CXP
<i>Corynebacterium</i> spp.	26	6	24	23	15	26	26	26
<i>Escherichia coli</i>	81	19	73	0	75	58	58	14
<i>Klebsiella</i> spp.	10	2	10	0	8	8	8	0
<i>Serratia</i> spp.	46	11	2	2	8	5	5	2
Other Gram-negative spp.	17	4	7	3	13	12	12	3
<i>Staphylococcus aureus</i>	50	12	49	19	40	45	45	49
<i>Staphylococcus</i> spp.	19	5	19	16	18	19	19	19
<i>Streptococcus agalactiae</i>	12	3	12	12	0	12	12	12
<i>S. dysgalactiae</i>	55	11	55	55	44	55	55	55
<i>S. uberis</i>	112	25	112	112	20	112	112	112
Other Gram-positive spp.	11	2	10	9	10	11	11	11
Totals	439		373	251	251	363	363	303
Percentage	100		85	57	57	83	83	69

^a CXM, cefuroxime 30 µg/disc; P, penicillin 10 i.u./disc; S, streptomycin 25 µg/disc; SXP, streptomycin + penicillin 50 µg + 10 i.u./disc; CXP, cephexazole + penicillin 25 µg + 25 i.u./disc.

on penicillin against β -lactamase produced by Gram-negative organisms. The production of β -lactamase by these strains was confirmed using nitrocefin.

Serratia species was the only major group of pathogens not sensitive to cefuroxime. However, 89% of the strains were isolated from one herd which provided 17% of all the samples, and as a result the collection of pathogens in this study may include an atypically high percentage of *Serratia* spp. If the *Serratia* strains were excluded from the analysis, 94% of all the remaining organisms would be sensitive to cefuroxime, 63% sensitive to penicillin, 62% sensitive to streptomycin, 91% sensitive to Streptopen and 77% sensitive to Cepoxillin.

Conclusions

Penicillin was successfully used for the treatment of bovine mastitis for more than 25 years before penicillin-resistant staphylococci became more common as mastitis pathogens, and the numbers of Gram-negative strains implicated also increased. The introduction of cephalosporins and chemically modified penicillins, the use of combinations of aminoglycosides and β -lactam antibiotics and the introduction of high-potency preparations were all in response to the changing aetiology of the disease. Some of these changes carried the penalty of increased milk-discard times. The results available suggest that the so-called 'second generation' cephalosporins may offer effective single-antibiotic treatment of mastitis combined with shorter milk-discard times.

Acknowledgements

We are grateful to our colleagues Mr R.B. Harding, Mr R. Joby and Mr P.R.D. Hardy, who contacted the veterinarians and arranged for the supply of samples.

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CHICK SALMONELLA INFECTIONS—COMBINED THERAPY BY ANTIMICROBIALS AND INTESTINAL BACTERIA

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Introduction

Poultry are a major source of human salmonella infections. There is a paucity of reliable data, based on statistically designed sampling procedures and uniform methodology, that gives the incidence of salmonella-contaminated poultry carcasses. A recent review indicates that the incidence is usually in the range of 25–65%, but lower in some countries with less than 1% in Sweden, about 5% in Denmark and about 7% in Finland. There is also a high correlation between the serotypes most frequently found in poultry and those causing infections of humans. Symptomless salmonella infection in chicks is a problem which cannot be solved completely, either by hygienic procedures or by the use of antimicrobials.

In the beginning of the 1970s, studies at the National Veterinary Institute, in Finland, demonstrated that the probable basis of the susceptibility of the broiler chickens was the delayed establishment of a normal intestinal flora in chickens reared according to modern mass production methods (Nurmi and Rantala, 1973). Furthermore it was shown that salmonella infection could be prevented by feeding the chickens with anaerobic cultures of normal intestinal adult fowl flora. The idea which we published 1973 has been confirmed by several research workers in different countries (e.g. Snoeyenbos, Weinack and Smyser, 1978; Barnes and Impey, 1980; Pivnick, Blanchfield and D'Aoust, 1981) and was the basis for the following concept (Pivnick and Nurmi, 1982): Nurmi, 1982):

- (1) Newly hatched chicks may be infected by a single cell of salmonella.
- (2) Older birds are resistant to infection because of the autochthonous microbiota of the gut, particularly the caeca and colon, but possibly other portions of the gut.
- (3) Chicks hatched by sitting hens are probably populated rapidly by the autochthonous gut microflora of the adult.
- (4) Hatcheries have replaced sitting hens, however, and the mass production of chicks is carried out in such a sanitary environment that the autochthonous microflora is not introduced at the modern hatchery.
- (5) The rearing barns in which the newly hatched chicks are placed are

usually sanitized and the floors covered with fresh litter between crops of chickens. Thus, autochthonous flora of adult birds are not readily available to populate the gut of the newly hatched chicks.

- (6) The introduction of intestinal microflora of adult birds to newly hatched chicks makes most of them immediately resistant to 10^3 – 10^6 infectious doses of salmonella.
- (7) The intestinal flora of adult birds may be introduced as a suspension of faecal droppings, caecal material or anaerobic cultures: these are designed as 'treatments'. Treatments may be introduced directly into the crop or by addition to the drinking water and possibly to the feed. Aerosols may also be useful vectors.
- (8) The source of the treatment is the homologous species, although treatment derived from chicken protects turkeys and vice versa.

The cultures of intestinal flora effective for prevention of salmonella infections have so far been mixtures of many bacteria. Anaerobic bacteria and conditions have been found essential for preventive capacity. In the product developed today, there should be about 20 different intestinal bacteria which achieves the preventive effect against salmonella, but also against infections such as *Escherichia coli* and *Campylobacter* spp. As is known, *Campylobacter* is becoming of more and more importance as a causative agent in food-borne infections in man.

As mentioned before, symptomless salmonella infection in broilers is a problem, which cannot be solved completely, either by hygienic procedures or by the use of antimicrobials. Antimicrobials cannot be used just before slaughter, because of the undesirable effects of the residues on human health. A complete cure of the flock is not usually achieved by the use of antibiotics and salmonella infection reappears soon after stopping the therapy and spreads rapidly in the chicken flock. That is mainly because the therapeutic antimicrobials destroy the normal intestinal flora and thereby increase the sensitivity of the chicken to the salmonella infection. Different antimicrobial agents have been used both prophylactically and therapeutically against salmonella infections and variable results have been reported. In order to avoid the negative effect of antibiotics destroying normal intestinal flora, experiments have been made combining antimicrobial therapy with giving broilers, after treatment, mixed cultures of intestinal bacteria.

One experiment was carried out as follows (Seuna and Nurmi, 1979; Seuna *et al.*, 1980): One-day-old chickens were infected with *Salmonella infantis*. The antimicrobial therapy, when given, was started 6 days later and lasted for 7 days. One test group in every trial was given only the antimicrobials and was killed and examined immediately after withdrawal of the drugs. A second group was treated in the same way, but examined 1 week after withdrawal of the drugs. The third series consisted of groups given various antimicrobial drugs, and the protective culture on 3 successive days after withdrawal of the antimicrobials. The fourth series consisted of groups given only the protective culture at the same time as the third series. The chickens of the third and fourth series were killed at the age of 3 weeks. The control groups were only infected with *S. infantis* and were examined for salmonellae at the same time as the test groups.

Results from experiments using combined therapy by antimicrobials and intestinal flora

The following drugs were used in the first experiments: (a) neomycin/polymyxin, (b) oxytetracycline/neomycin, (c) dihydrostreptomycin, (d) furazolidone, (e) trimethoprim/sulphadiazine. The oxytetracycline/neomycin therapy was most effective, but reappearance of the infection was not avoided. Generally, combined therapy with other antimicrobials and the culture reduced the number of infected chicks compared with the respective control group. The combination polymyxin/neomycin was particularly effective.

In the second experiment, the following antibiotics and antimicrobials were used: (a) neomycin, (b) neomycin/oxytetracycline, (c) neomycin/polymyxin, (d) sulphadiazine/trimethoprim. In this experiment the combined therapy with oxytetracycline plus neomycin and bacterial culture seemed to be most effective. Practical experiment with combined therapy was performed in a grandparent flock of 4000 chickens imported from the USA and infected with *S. infantis* (Seuna, Andersson and Nurmi, 1978). The first isolations were made from faecal samples when the chickens were at the age of 10 days. The first isolation was made at the breeders' laboratory and confirmed at the National Veterinary Institute.

When the chickens were 5 weeks old, they were treated with 110 mg of oxytetracycline and 77 mg of neomycin per litre of drinking water for 13 days. On the first, second and third days after the antibiotics were withdrawn the chickens were treated with an anaerobically incubated intestinal culture. The purpose of the treatment with bacteria was to re-establish the normal intestinal flora and to prevent reinfection. One day before the antibiotics were withdrawn, the surface of the litter was removed and replaced with new litter. The equipment and the litter were disinfected with 0.5% Des L 14 solution (TAD Pharmaceutisches Werk GMBH, West Germany) and 3 weeks later with 1% Lysovet (Farmos Incorporated, Turku, Finland).

The sampling was started 2 weeks after the treatment. Until the beginning of the laying period, 31 chickens were killed and their intestines examined for salmonellae. In addition, 81 samples of faeces taken randomly from the chicken house were examined. All the samples were salmonella-negative. In addition to this official sampling, 21 samples were examined at the breeder's laboratory. *Salmonella infantis* was isolated from one sample of dust.

The first lot of 50 eggs laid by the flock was hatched. One whole egg, one sample of shells and 28 chickens were examined and found to be free from salmonella. Neither were salmonellae isolated from the samples of the next lot of 10 000 eggs. All samples taken during the laying period and later at slaughter were also salmonella-negative.

Antibiotics are added when permitted to feed at low levels, about 15–25 p.p.m., to stimulate growth and at high levels, 100–200 p.p.m., for therapy against various bacterial diseases. High levels of some antibiotics given in feed or water may suppress salmonella infections in flocks, but do not eliminate them, when antibiotic treatment is terminated. Salmonellae are excreted again. Two questions have arisen: (a) do antibiotics in the feed destroy the protective flora, and (b) can infected flocks be cleared of salmonellae by a combined therapy of antibiotics followed by intestinal culture?

Many antibiotics added to feed do not destroy the protective effect of

treatment. Chicks treated with faecal microflora and fed with antibiotic-containing feed, usually at 200 p.p.m. (10 p.p.m. of nitrovin), resisted subsequent challenge with salmonella. The antibiotics were bacitracin, furazolidine, gallimycin, penicillin/streptomycin, chlortetracycline and tylosin (Nurmi and Rantala, 1974; Snoeyenbos, Weinack and Smyser, 1979).

Medication of infected older birds, for about 1 week, with antibiotics to decrease the incidence and intensity of infection and subsequent introduction of faecal culture on several successive days has apparently eliminated infection (Seuna, Andersson and Nurmi, 1978) or decreased the number of infected chicks. In those chicks that remained infected, this combined treatment decreased the number of salmonellae per gram of faeces (Seuna and Nurmi, 1979; Seuna *et al.*, 1980). The best antibiotics for the combined therapy were mixtures of polymyxin and neomycin or neomycin and oxytetracycline. Such therapy may be useful when breeder flocks are infected, or when broiler flocks are infected and salmonella-contaminated meat is not acceptable to regulatory authorities and on the whole should be avoided because of hazards to public health.

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ARE RESISTANT GRAM-POSITIVE BACTERIA IN ANIMALS A THREAT TO MAN?

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Introduction

There is little doubt that antibiotic use in both man and animals selects antibiotic resistance (Hummel and Witte, 1981). By manipulations *in vitro*, it is possible to extract genes coding for resistance from one bacterial species and insert them into many others. Thus, the use of antibiotics in animals can select resistance, and the resistant progeny have the potential to colonize or infect man, with their genetic determinants possibly becoming established in the 'human' flora. These risks have been well publicized in Gram-negative bacteria, notably *Salmonella typhimurium*. Before leaving this subject it is worth commenting that the occurrence of these postulated events under natural conditions is rare (Lacey, 1981a).

There has also been speculation that the use of antibiotics active against mainly Gram-positive organisms in animals poses therapeutic problems in clinical medicine. Briefly, the argument proceeds as follows (Novick, 1981; Dunny *et al.*, 1981):

- (1) Antibiotics used in veterinary medicine and in agriculture, particularly if used as feed additives, select resistance in animal bacteria.
- (2) There is opportunity for man to be colonized or infected by these resistant organisms through, for example, husbandry, slaughtering, preparation and consumption of food.
- (3) Those resistant strains might pose therapeutic problems themselves, or the genes in question may contribute to the total reservoir of resistance.

It has not so far been possible to refute or substantiate these possibilities, mainly because of the absence of stable markers to identify the bacterial clones that might be involved, and also, in most instances, the relevant transmissible genes. Unfortunately, many of our attitudes over the past two decades have been based on the assumption that certain manipulations *in vitro* reflect naturally occurring events (Swann Committee, 1969). In the analysis that follows, it is not intended to imply that antibiotic use in animals never causes problems for treating Gram-positive infections in man, but that the contribution of animal resistant cultures directly or indirectly is at present negligible. Perhaps if the human use of antibiotics does so improve to reduce

the selection of resistance in 'human' strains to minimal amounts, then antibiotic use in animals might become, proportionally, a more significant contribution to the total incidence of resistance. First, it is pertinent to consider the origins of antibiotic resistance.

Development of antibiotic resistance in *Staphylococcus aureus* of human origin

Apart from resistance to penicillin (by production of β -lactamase) in perhaps 1–2% of human *Staphylococcus aureus* isolates in the early 1940s, cultures then were uniformly sensitive to the antibiotics now available. However, resistance has since developed to all the major anti-staphylococcal agents, except to the relatively little used vancomycin. It is possible to account for the emergence of the contemporary multi-resistant clones by the following events (Lacey, 1975). A minority of antibiotic resistance, i.e. to novobiocin, rifampicin, streptomycin and fusidic acid (resistance to the latter can also be plasmid mediated) appears to be via classical mutation, whereby a cell containing a random point change in its chromosomal DNA is selected by antibiotic exposure. The contribution of this type of resistance is small for two reasons; first, many mutants are defective and have reduced viability, and secondly, once an antibiotic has been found to be associated with such a mutational risk, it has been used sparingly.

Most natural antibiotic resistance is determined by relatively large sequences of DNA—perhaps 1–5 million daltons—that contain specific control and structural genes. These resistance sequences are commonly carried by plasmids, although they can sometimes be chromosomal, presumably as a result of transposition (Novick *et al.*, 1979; Phillips and Novick, 1979). 'Methicillin resistance' is phenotypically and genetically unique; the relevant genes are probably carried on an additional chromosomal linkage group that has no comparable structure in sensitive cells (Kuhl, Pattee and Baldwin, 1978; Stewart and Rosenblum, 1980).

During the late 1960s and the early 1970s, multi-resistant clones of *S. aureus* carried a variety of plasmids, each characteristically determining resistance to single antibiotics. However, some recently isolated multi-resistant staphylococci harbour surprisingly few plasmids, presumably as a result of transition (in the general sense) of some of the plasmid genes into the chromosomes (Lyon *et al.*, 1982). This does not invalidate the general hypothesis that multi-resistant staphylococci have acquired plasmids by successive transfer events. Thus, in the evolution of the multi-resistant staphylococcus it is important to identify the source of these plasmids. Potential donors include other strains of *S. aureus*, coagulase-negative staphylococci, possibly streptococci or even other genera.

There is strong evidence that prophages have spread within the natural staphylococcal population (Rosendal and Bülow, 1964). Gain of prophage is associated with resistance to lysis by that and related phages (lysogenic immunity); this explains why multi-resistant isolates are lysed by few, if any, typing phages. Other causes of phage immunity include restriction/modification (Stobberingh and Winkler, 1976; 1977), essentially a characteristic of group II strains. These are in many ways distinct from the mainstream

evolution of the organism. Deficiencies in cell wall teichoic acid can cause failure of phage absorption (Chatterjee, 1969). This seems rare because most non-typable organisms appear to absorb the relevant phage. Study of multi-resistant staphylococci from Australia (Gedney and Lacey, 1982) shows that their resistance to lysis by typing phages is not due to restriction/modification nor defect in phage absorption (R. W. Lacey, unpublished observations). This implies that lysogenic immunity is the cause of their narrow typing spectrum. Thus, multi-resistant staphylococci show two major differences from earlier sensitive strains: the presence of both novel prophages and resistance genes, commonly carried by plasmids. Using mutagenesis, it is possible to convert modern non-typable strains to types isolated in earlier years (i.e. strains in group I with or without multiple reactions in group III). The mechanism of this change is presumably by deletion of part or all of the genes responsible for lysogenic immunity (Lacey and Stokes, 1979). This is consistent with the proposal that cultures isolated recently from clinical material have evolved from the earlier human cultures. Methicillin resistance provides an excellent marker for such studies, as these cells also possess stable chromosomal streptomycin resistance (Lacey and Grinsted, 1973).

Carriage of novel prophages (perhaps defective) and genes specifying antibiotic resistance are therefore often associated, and presumably of value to the cell. Prophage carriage could benefit the recipient cell by protecting it during generalized transduction, and to permit conjugation between donor and recipient cells (Lacey, 1980a). Co-incubation of a marked derivative (the potential recipient) of *S. aureus* 1030 with a clone of 1030 containing a plasmid coding for tetracycline resistance, never results in transfer (1030 is non-lysogenic, contains no plasmids and no detectable restriction/modification system). When any one of a variety of bacteriophages are inserted into the recipient 1030, transfer from the donor readily occurs, too soon for 'back-transduction'. The molecular events are not fully known, but the presence of the prophage does not apparently cause aggregation of the cells (R. W. Lacey, unpublished observations). Conjugation is likely to be more important in nature than transduction in *S. aureus*, because it is not associated with destruction of either the donor or the recipient. Transducing particles are apparently formed only after death of the donor and many potential recipients may be lysed by 'normal' phages. Moreover, transduction occurs at lower frequencies than conjugation.

Experiments *in vitro* have attempted to identify the source of plasmids in human *S. aureus*. Many workers have published findings where transfer of resistance has been detected, but it is not known how many negative results remain unpublished. From unpublished work we can conclude:

- (1) In *S. aureus* to *S. aureus* matings, the highest frequency of plasmid transfer *in vitro* occurs between different derivatives of the same culture, e.g. 8325 (Novick, 1967) and 1030 (Lacey, 1980a).
- (2) Many workers have failed to transfer resistance from *S. epidermidis* to *S. aureus*. Where transfer has been detected it occurs at low frequency—c. 10^{-8} (Jaffe *et al.*, 1982); it is uncertain whether such transfer frequencies will indicate the probability of transfer *in vivo*. It may also be difficult to define certain cultures as either *S. aureus* or *S. epidermidis*. Thus, very accurate information is needed in the identification of the organism and

- its derivatives before it can be accepted that resistance has been transferred interspecifically.
- (3) It is rare for resistance to transfer from animal strains to human in mixed cultures or by transduction; similarly, resistance is not transferred readily from human to animal (Lacey, 1980b). However, once the appropriate genes have become established in a new host, further transfer within that host—whether animal or human—occurs at higher frequencies.
 - (4) Although transfection (transformation with bacteriophage DNA) or transformation can cause the insertion of DNA into human *S. aureus* from a variety of donor bacterial species, highly artificial conditions are required, including inhibition of the naturally occurring DNA'se enzymes.
 - (5) Generally, spontaneous transfer of resistance can be expected to occur most readily in nature between clones of similar cultures, with little reason to suppose that transfer from animal to human cultures will be common.

Decline in the incidence of the multi-resistant *Staphylococcus aureus*

Monitoring of the incidence of resistant bacteria over the long term is beset with difficulties, not least because it is virtually impossible to standardize the nature of the specimen referred for culture. For example, the incidence of resistance may well appear low if all specimens were referred for culture prior to therapy, and would be high if specimens were obtained selectively from patients after therapeutic failure. Despite this, there is consensus that the incidence of multi-resistant staphylococci is declining within the hospitals of the UK. However, in Greece (Giamerellou, Papapetropoulou and Daikos, 1981), Australia (Gedney and Lacey, 1982), Dublin (Cafferkey *et al.*, 1983) and probably in the USA (Eickoff, 1982), considerable morbidity and mortality are caused by multi-resistant staphylococci.

The cause of the decrease in resistant organisms is probably multifactorial; for example, better use of chemotherapeutic agents, reduced growth rate of the resistant organisms (Lacey and Chopra, 1975) and the instability of the plasmids involved. Relevant to this is the finding that, on storage *in vitro*, the frequency of transfer of resistance to several antibiotics from donors to standard recipients declines (*Table 17.1*). Carefully purified clones of the

Table 17.1 EFFECT OF STORAGE ON FREQUENCY OF TRANSFER OF SOME STAPHYLOCOCCAL PLASMIDS IN MIXED CULTURES^a

Donor culture	Recipient	Plasmid coding for resistance	Mol. wt ($\times 10^6$ dalton)	Frequency on first construction of strains (1979)	Frequency after 4 years storage
1030	1030(55)	Tetracycline (<i>tet-r</i>)	3.0	3.2×10^{-2}	2.77×10^{-5}
1030	1030(55)	Neomycin (<i>neo-r</i>)	5.0	3.6×10^{-4}	6.7×10^{-7}
1030	1030(55)	Cadmium (<i>cp-1</i>)	21.0	2.4×10^{-5}	4.3×10^{-7}
1030	1030(55)	Cadmium / penicillin (<i>pen-r</i>)	19.5	5.7×10^{-5}	6.4×10^{-7}
1030	1030 S	Cadmium (<i>cp-2</i>)	22.0	1.8×10^{-3}	5.6×10^{-8}
1030	1030 S	Cadmium (<i>cp-3</i>)	21.0	7.0×10^{-3}	5.4×10^{-4}

^a See Lacey (1980b) for details of cultures and strains.

stored cultures still possess plasmid DNA, but are present in fewer copies/chromosome compared to that in the originally constructed strain (R.W. Lacey, unpublished observations). A selective disadvantage would be anticipated from the possession of many copies of a plasmid, since just one or a very few copies must be functional as transduction will not introduce large numbers of copies at any one procedure. Presumably the reduced number of copies of the plasmid explains the reduced transfer frequency.

Furthermore, when a cell is constructed to possess many different plasmids, the amount of plasmid DNA is less than additive, and some of the plasmids show decreased stability (Lacey and Chopra, 1974). These observations suggest that antibiotic resistance in *S. aureus* is potentially reversible. Any gain of antibiotic resistance, for example, from animal cultures, must be viewed in the light that such elements are, to varying extents, unstable.

Antibiotic resistance in streptococci

The genus *Streptococcus* includes an enormous variety of species and types. Study of the mechanism of resistance in one species cannot necessarily be related to another. All the three 'classical' mechanisms of gene transfer—transformation, transduction and conjugation—can occur in streptococci *in vitro* (Clewell, 1981), but it is not possible to manipulate the genes of any one strain by more than one or possibly two of these mechanisms. Any assumptions concerning the spread of resistance between different species must therefore be based on strong evidence.

The mechanism of resistance transfer in group D streptococci has been studied in recent years (see the excellent review by Clewell, 1981). Many isolates of group D streptococci from clinical material are resistant to antibiotics (but rarely to ampicillin or nitrofurantoin). Most of the resistance is plasmid mediated, but tetracycline resistance may be determined by chromosomal genes capable of transfer during mating experiments ('conjugative transposons', Clewell, 1981). Two types of conjugation occur in this organism (Clewell, 1981). One occurs during high cell-to-cell contact that can be achieved on filters or on the surface of agar. The other occurs in broth cultures (Jacob and Hobbs, 1974), where the potential recipients secrete a peptide (clumping inducing agent or 'CIA') that causes donor cells to adhere and plasmids to pass from donor to recipients (Clewell, 1981). Individual recipients may secrete a variety of CIAs differing in specificity for different plasmids in the donor. Establishment of the plasmids in the recipient inhibits the synthesis of the corresponding CIA. Thus, many cultures of group D streptococci from the human intestinal tract produce CIAs, possess plasmids and are resistant to antibiotics (Dunny *et al.*, 1979). *In vitro*, it is possible to transfer resistance from these group D streptococci to other strains, notably those of group B (van Embden, Engel and van Klingeren, 1977; Malke, 1979). Transformation and transduction have not been performed in group D streptococci, but in the clinically important group A streptococcus, antibiotic resistance can be transferred by transduction (Malke, 1972), but not by conjugation. The notion that group D streptococci provide a reservoir of resistance determinants capable of transfer to group A cultures is probably mistaken. It is possible to transfer various determinants between pneumococci

(Smith *et al.*, 1980), streptococci and staphylococci and even between streptococci and some Gram-positive bacilli using highly artificial conditions; the mechanisms vary (Hershfield, 1979; Gibson *et al.*, 1979; Engel *et al.*, 1980).

The current incidence of resistance in streptococci causing infection in man is variable; macrolide resistance in group A streptococci is high in Japan (Miyamoto *et al.*, 1979) but low elsewhere, and earlier fears that penicillin resistance would become common in pneumococci (Jacobs *et al.*, 1978), have not materialized—at least not yet. So far, no pathogenic streptococcus has been isolated that produces β -lactamase. Our own figures of resistance in this organism are shown in Table 17.2.

Table 17.2 INCIDENCE OF ANTIBIOTIC RESISTANCE IN SOME STREPTOCOCCI ISOLATED IN KING'S LYNN, 1980

Culture	No. tested	Penicillin	No. resistant to:		Cephadrine
			Erythromycin	Tetracycline	
<i>Streptococcus pneumoniae</i>	235	0	0	8 (3.4%)	0
Group A	319	0	0	14 (4.4%)	0
Group C	62	0	0	13 (21.0%)	0
Group G	106	0	0	9 (8.5%)	0
Total	722	0	0	44 (6.1%)	0

Macrolide resistance in staphylococci and streptococci

The use of macrolide antibiotics, particularly tylosin, as growth promoters in animal husbandry has provoked concern that they will directly or indirectly jeopardize therapy in man (Novick, 1981; Dunny *et al.*, 1981). The nature of macrolide resistance therefore requires careful scrutiny. The two common phenotypes of macrolide resistance in both staphylococci and streptococci are (1) inducible or (2) constitutive.

The inducible type has been known as dissociated (Garrod, 1957), because erythromycin exposure induces resistance to all macrolides, lincosamines and streptogramin B antibiotics (known collectively as MLS antibiotics). The inducible resistance is easily demonstrated by placing discs containing erythromycin and lincomycin on plates seeded with such a culture. After incubation, erythromycin appears to antagonize the inhibitory action of lincomycin.

In the constitutive type of resistance there is resistance to all MLS antibiotics without prior antibiotic exposure. There are a number of variants of the constitutive type of resistance that can be low level or incomplete (e.g. Dutta and Devriese, 1982). In addition to these common mechanisms, streptococci (but apparently not staphylococci) may be resistant to lincomycin alone, sometimes through the ability of animal cultures to hydrolyse the antibiotic (Dutta and Devriese, 1982). The biochemical mechanism of macrolide resistance is methylation of ribosomal RNA, so that erythromycin no longer inhibits protein synthesis (Weisblum, 1975).

In *S. aureus*, the relationship between the common phenotypes appears clear cut. In both clinical studies and *in vitro*, the inducible phenotype alters

apparently irreversibly to the constitutive through a point mutation at a locus near the genes determining inducible resistance (Lacey, Lewis and Rosdahl, 1974).

As might be expected, the incidence of the various MLS types varies with the selection pressure. Of these antibiotics, only erythromycin is now used commonly in clinical medicine in the UK. Almost all macrolide-resistant staphylococci isolated over the past two years in the King's Lynn Health District, in the UK, are inducibly resistant (*Table 17.3*). In contrast, macrolide-resistant cultures from animals where the selection pressure includes tylosin are characteristically constitutively resistant to all the MLS antibiotics (Lacey, 1980b). That the MLS phenotypes are different in animal and human staphylococci argues against the importance of an 'animal reservoir' of resistance genes capable of infecting human cultures.

Table 17.3 NATURE OF ERYTHROMYCIN RESISTANCE IN CULTURES OF *Staphylococcus aureus* ISOLATED DURING 1981 AND 1982, FROM HOSPITAL AND NON-HOSPITAL SOURCES, KING'S LYNN, UK

No. strains with inducible resistance to erythromycin only (dissociated resistance)	79
No. strains with full constitutive resistance to erythromycin, spiramycin, tylosin, lincomycin, clindamycin (MIC > 100 µg/ml)	2
No. strains with partial constitutive resistance to all macrolides and lincomycins (MIC 10–100 µg/ml)	2

Among streptococci from human sources, MLS resistance is often determined by plasmids of mol. wt $15\text{--}20 \times 10^6$ dalton (Hershfield, 1979; Clewell, 1981). Streptococcal and staphylococcal plasmids coding for resistance to MLS antibiotics possess similar sequences (Weisblum, Holder and Halling, 1979). These plasmids are thus found in dissimilar species between which it is difficult, if not impossible, to exchange genetic information. Moreover, there is a common DNA sequence in many of these that determines MLS resistance. Explanations of this are conjectural, but the possibility of interspecies transfer cannot be excluded. Perhaps it is more likely that the various Gram-positive organisms resistant to MLS antibiotics have differentiated from a common ancestor into distinct species. The ancestor may be related to *Streptomyces erythreus* itself (the source of erythromycin!). Thus, while specific DNA sequences can be found on similar sized plasmids in a variety of bacterial species, the extent, and the direction, of any transfer of the genes in recent years are not known.

Many studies attempting gene transfer with negative results may not be reported. Because of these uncertainties, a systematic study has been performed searching for gene transfer within and between animal and human streptococci.

Attempt at transfer macrolide resistance between animal and human streptococci

POTENTIAL DONOR CULTURES

Sixty-eight macrolide-resistant animal isolates were employed. These had been isolated from pigs and cattle. Each was judged to be epidemiologically

distinct. These were all constitutively resistant to all the MLS antibiotics except for two that degraded clindamycin (Dutta and Devreise, 1982). Thirty distinct human isolates of macrolide-resistant group D streptococci were isolated from fresh faecal or urinary specimens.

POTENTIAL RECIPIENTS

Twenty miscellaneous animal streptococci (groups A,B,C,E,P,L,S) and 12 group D streptococci from pigs and cattle were used. Human recipient cultures included 20 group A *Streptococcus pyogenes*, 12 group B, 10 group C and 24 group D. Resistant recipients were usually detected by selecting with 5 µg erythromycin/ml with either 50 µg rifampicin, 5 µg fusidic acid or 100 µg streptomycin/ml.

Each set of transfer experiments included a donor culture known to transfer resistance to the recipient (M439, 2390 or K2852, kindly supplied by Dr J. van Embden). Controls also included each donor and recipient incubated singly. Where low frequency transfer was thought to have occurred ($\sim 10^{-7}$ – 10^{-8}), the experiment was repeated several times. In experiments where transfer occurred, a proportion of the presumptive resistant recipients were examined to identify the direction of transfer.

Results

In nutrient broth, the incidence and frequency of transfer to and from groups B and D were similar; the results were combined (*Table 17.4*). Groups A or C did not acquire macrolide resistance from any animal or human donor. The majority of animal streptococci failed to transfer their resistance to human recipients; in about 10%, a low frequency transfer was detected, and in only $\sim 1\%$ of the matings, was a frequency of $> 10^{-6}$ obtained. However, transfer of resistance from animal group D cultures occurred more readily to other animal streptococci (but not to group A or C), so that transfer occurred in about one-third of the matings. When human donors were employed, a comparable pattern emerged: human cultures transferred their resistance to animal donors occasionally and at low frequency, but the transfer occurred more readily to human recipients, although the frequency of transfer was usually low. Overall, from 5816 matings, transfer occurred between 684 (11.8%) pairs. But in only 111 (1.9%) was the transfer frequency $> 10^{-6}$ (*Table 17.4*).

Because maximum frequency of resistance transfer between streptococci may occur under conditions of high cell density (Clewell, 1981), donors and recipients were cultivated directly on the surface of blood agar or on filters (0.45 µm pore size). Sixteen animal group D donors and 12 human group D recipients were selected between which transfer of resistance had not been detected in nutrient broth (frequency $> 10^{-8}$). Appropriate controls included a pair of group D cultures between which transfer frequency was increased by about 3 logarithms after co-incubation on filters compared to that in broth. In only one mixture did transfer occur on filter papers (frequency $\sim 10^{-5}$) but not in broth.

Table 17.4 ATTEMPT AT TRANSFER OF MLS RESISTANCE BETWEEN ANIMAL AND HUMAN STREPTOCOCCI IN NUTRIENT BROTH

Donor source	Streptococcal group of donor	Recipient source	Streptococcal group of recipient	Absence of transfer ^a	Percentage transfer	
					Frequency 10 ⁻⁶ - 10 ⁻⁸	Frequency > 10 ⁻⁶
Animal	D 36	Human	A (20) C (10)	1080 ^b (100%)	0	0
Animal	D (36)	Human	D (24)	726 (84.0%)	130 (15.0)	8 (0.9)
Animal	A (3) B (9) C (3) E (6) NG (11)	Human	B (12) D (18)	854 (88.9%)	94 (9.8)	12 (1.2)
Animal	A (3) B (9) C (3) E (6) NG (11)	Human	A (10) B (6)	512 (100%)	0	0
Animal	D (36)	Animal	A (2) B (3) C (4) E (3) P (2) L (4) S (2)	476 (66.1%)	173 (24.0)	71 (9.8)
Human	D (30)	Animal	A (2) B (3) C (4) E (3) P (2) L (4) S (2)	561 (93.5%)	36 (6.0)	3 (0.5)
Human	D (10)	Animal	D (12)	110 (91.6%)	10 (8.3)	0
Human	D (30)	Human	A (6) C (4)	300 (100%)	0	0
Human	D (30)	Human	B (6) D (16)	513 (77.7%)	130 (19.6)	17 (2.5)

^a Resistant recipients/total recipients.^b Numbers refer to total numbers of recipient/donor matings.

SURVIVAL AND PATHOGENICITY OF ANIMAL CULTURES IN MAN

The above findings suggest that genes determining resistance within the animal staphylococcus and streptococcal cells will only rarely spread to streptococci and staphylococci of clinical importance. Even the risk of resistance of animal group D streptococci becoming established in human group D cultures will have little impact on chemotherapy in man, because the resistance should not 'spread' further in groups A or C and the two resistance determinants most likely to be involved—those coding for tetracycline and erythromycin resistance are rarely used in treating urinary infections, by far the commonest cause of sepsis due to group D streptococci. Indeed, one of

the therapeutic advantages attributed to erythromycin is that it does not select resistance in common urinary pathogens!

But what are the risks of resistant animal Gram-positive organisms causing therapeutic problems themselves for man? The answer must virtually be none, since most staphylococci and streptococci are highly adapted to individual mammalian hosts (Gibbons, Spinnel and Skobe, 1976). Reports of animal streptococci causing infection in man are exceedingly uncommon. For example, Kurl (1981) acquired an acute pharyngitis during laboratory manipulations of an animal culture. But other instances are rare indeed. As far as staphylococci are concerned, there are few instances when similar strains have colonized man and cattle (Wallace *et al.*, 1962; Thawley *et al.*, 1977), but these are typically 'human' isolates, so the direction of spread is likely to be from man to animals rather than vice versa (Hummel and Witte, 1981).

During the 1960s some epidemic strains of *S. aureus* caused problems in hospitals in the UK. These cultures were typically neomycin resistant and produced a characteristic lemon-yellow pigment on appropriate solid media; the origin of these cultures was obscure—one explanation offered was an animal origin (Jacobs and Willis, 1964). However, it is easy to isolate such yellow pigment variants following mutagenesis of the classical golden type (Grinsted and Lacey, 1973), so these strains could well be derived from the human staphylococcus. Animal cultures of *S. aureus* do survive badly in simulated human experiments—notably after application to human skin, in human urine and blood (Lacey, 1981a).

Discussion

The question as to whether resistant Gram-positive bacteria (essentially staphylococci and streptococci) pose problems in man has important commercial and practical considerations. It is not possible to answer this question categorically in one way or the other. The contribution of genes coding for resistance selected by antibiotic use in animals to the general pool in 'human' pathogens must vary with the relative use of antibiotics in the two groups. Where animal use of antibiotics is large and medical use small, then resistant animal organisms might pose a significant contribution to the overall amount of resistance in human pathogens. But where human use exceeds animal by a large amount, then the contribution of animal use must be small. Accurate figures are not available, but in the UK at present, the veterinary and agricultural use of antibiotics probably account for about one-quarter of total antibiotic consumption. In other countries where antibiotics are more easily available or more readily prescribed in human medicine, animal use may comprise an even smaller component of total use.

Therefore, if antibiotic resistance is to be controllable by legislative procedures, the approach should be principally towards human use. This does not imply that there should not be continual monitoring of the agricultural and veterinary use of antibiotics. If these uses do increase substantially, then concern that medical use of antibiotics is being threatened may become justified. Animal use of antibiotics unquestionably selects resistance in animal cultures (Hummel and Witte, 1981; Dunny *et al.*, 1981), and these may well pose therapeutic problems in animals, although it would be interesting to

compare the virulence of resistant animal organisms with that of their sensitive equivalents. It is well known that the presence of additional DNA in the cell can be associated with reduction in growth rate of the cell (Lacey and Chopra, 1975) in antibiotic-free media. It is less commonly known that macrolide-resistant organisms grow slowly in the presence of antibiotics to which they are resistant (e.g. Lacey, 1981b). The clinical relevance of these observations is not known, because the fear that the observation *in vitro* (i.e. resistance) might be clinically important prevents use and evaluation of that antibiotic.

Thus, in general, the fears expressed by Novick (1981) and Dunny *et al.* (1981) that antibiotic use in animals, particularly the use of tylosin as a growth promoter, are a threat to human medicine, are at present unsubstantiated. Many powerful factors reduce the threat of resistant Gram-positive organisms from animals. First, considering the entire organism itself:

- (1) Streptococci and staphylococci are highly adapted to specific mammalian hosts. There are few types common to, for example, cattle and pigs and man. Where similar types of *S. aureus* have been isolated from man and animal, the direct spread is likely to be mainly from human to animals.
- (2) Experimentally, animal staphylococci survive badly in human environments.
- (3) The commonest resistance determinants—to tetracycline and erythromycin—in animal group D streptococci are of little relevance to medical therapy because these antibiotics are rarely used in the treatment of the commonest infection it produces, i.e. urinary infection.

Secondly, the genes from animal Gram-positive organisms can rarely become established in human cultures since:

- (1) It is very difficult to transfer resistance determinants from animal staphylococci to human.
- (2) The nature of resistance to several antibiotics in animal staphylococci differs from that in human. In particular, macrolide resistance in animal cultures, certainly those selected by the use of tylosin, are constitutively resistant to all the MLS antibiotics, while a recent survey in the UK shows that almost all macrolide resistance in clinical strains is of the inducible (or dissociated) type.
- (3) It is possible to account entirely for the emergence of the multi-resistant staphylococci by antibiotic use in man. In any event, the long-term viability of the multi-resistant organisms may well be reduced.
- (4) Resistance is common in both animal and human group D streptococci; it can be transferred readily between cultures *in vitro* by conjugation, but not by transduction nor transformation. This transfer may occur in nature, but transfer of resistance from animal cultures to human occurs much less readily than animal to animal or human to human.
- (5) It is not possible to transfer resistance *in vitro* from animal streptococci to the groups A or C streptococci from human sources.

Finally, it should be stressed that many surveys have shown that levels of resistance in human organisms are closely related to antibiotic use in man and shortcomings in cross-infection control. Reduction in resistance therefore

involves two main areas: (1) the use of antibiotics in such a way as to discourage resistance, e.g. avoid their topical use, and reduce the large numbers of patients who are treated unnecessarily, and (2) increase our efforts at confining resistant bacteria in hospitals.

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GENTAMICIN-RESISTANT SALMONELLAE IN TURKEY REARING

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Since the mid-1970s, salmonella-contaminated turkeys and turkey meat products have sometimes been the source of food-borne infections in the Federal Republic of Germany. In one epidemic, at a hospital, three people died. To prevent further outbreaks of salmonellosis, it was decided to dip hatching eggs into gentamicin solution and to inject 1-day-old chickens with gentamicin.

Since we expected that the prophylactic use of gentamicin would lead to the emergence of gentamicin-resistant salmonella, we tested all isolates, sent to the Veterinary Salmonella Centre, for gentamicin resistance. The number of strains isolated and their source are shown in *Table 18.1*. Their number increased from none in 1977 to 36 in 1980; the majority being isolated from turkeys and their environment.

Table 18.1 ORIGIN AND NUMBER OF GENTAMICIN-RESISTANT SALMONELLA STRAINS

	1978	1979	1980
No. of strains	3	14	36
Turkey		14	27
Environment			2
Chicken			1
Goose			1
Finch			1
Avocet (<i>Recurvirostra avosetta</i>)			1
Pig	2		
Dog	1		
Blood meal			3

Most of the strains were *Salmonella senftenberg* or related serotypes. Their total resistance profile is shown in *Table 18.2*. In order to learn more about the resistance genes and plasmids involved, we investigated all 14 isolates from 1979 more carefully.

Table 18.2 SEROTYPES AND RESISTANCE PATTERNS

Number of isolates	Serotype	Resistance pattern ^a									
4	<i>S. senftenberg</i>	Gn	Kn	Si	To	Str	Spc				
5	<i>S.E₄ O-Form</i>	Gn	Kn	Si	To	Str	Spc				
2	<i>S. saint paul 05 negative</i>	Gn	Kn	Si	To	Str	Spc	Ap			
1	<i>S. indiana</i>	Gn	Kn	Si	To	Str	Spc	Ap			
1	<i>S. indiana</i>	Gn	Kn	Si	To	Str	Spc		Tc	Cm	
1	<i>S. indiana</i>	Gn	Kn	Si	To	Str	Spc	Ap	Tc	Cm	

^aGn, gentamicin; Kn, kanamycin; Si, sisomicin; To, tobramycin; Str, streptomycin; Spc, spectinomycin; Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol

Plasmid profiles from 10 of these strains are shown in *Figure 18.1*. Using this approach we were able to assign each strain into one of six classes, defined by a distinct resistance pattern and plasmid DNA profile. A comparison of these classes is shown in *Figure 18.2*.

The predominating class is comprised of 9 strains, and its representative plasmid DNA profile is shown in the first track on the left. All strains belonging to this group are resistant to the aminoglycosides listed in *Table 18.2* and carry a 118 megadalton (Md) incompatibility group *IncH* plasmid. This type of aminoglycoside resistance will be called here gentamicin resistance.

The second class is similar to the first, but carries a small cryptic plasmid. The third and fourth class look very similar because both strains are

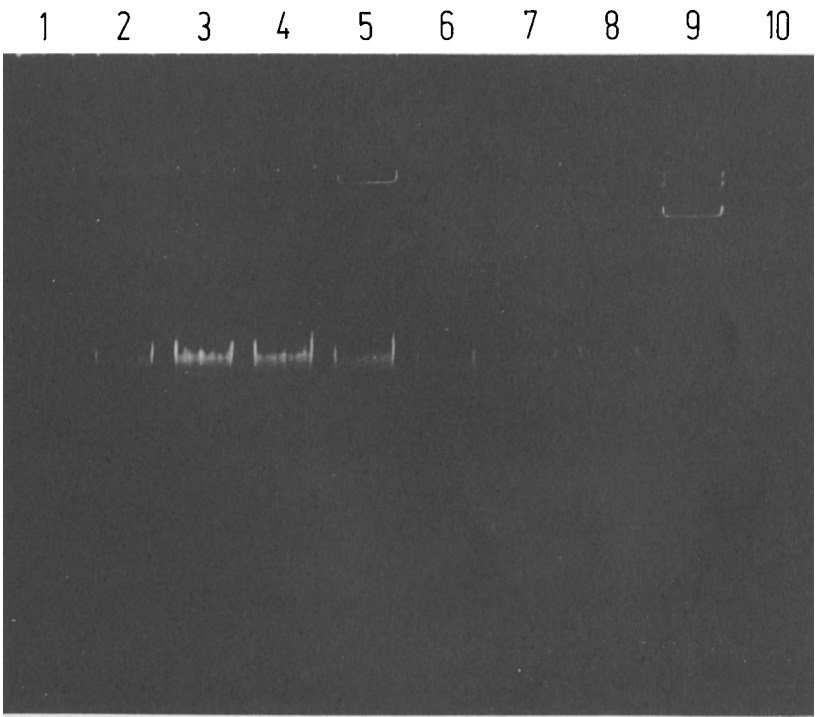


Figure 18.1 Plasmid DNA of 10 gentamicin-resistant salmonellae isolated in 1979

Table 18.3 TRANSFER FREQUENCY TO *Escherichia coli* K12 STRAIN BB1 IN LIQUID MATINGS

Strain	Resistance	Temperature (°C)	Transfer frequency obtained for: ^a			
			Gn	Ap	Tc	Cm
5316	Gn	37	$<1 \times 10^{-7}$			
		22	3×10^{-3}			
7448	Gn	37	$<8 \times 10^{-8}$			
		22	1×10^{-3}			
7444	Gn, Ap	37	$<2 \times 10^{-7}$	$<2 \times 10^{-7}$		
		22	$<2 \times 10^{-7}$	$<2 \times 10^{-7}$		
8580	Gn, Ap	37	7×10^{-7}	3.7×10^{-5}		
		22	$<1 \times 10^{-7}$	1.3×10^{-5}		
6690	Gn, Ap, Tc, Cm	37	1.4×10^{-1}	9×10^{-2}	1.7×10^{-5}	2×10^{-5}
		22	4.2×10^{-5}	3.8×10^{-5}	$<1.3 \times 10^{-7}$	$<1.3 \times 10^{-7}$
7446	Gn, Tc, Cm	37	$<2.2 \times 10^{-7}$		$<2.2 \times 10^{-7}$	$<2.2 \times 10^{-7}$
		22	1.3×10^{-5}		2.6×10^{-2}	2.0×10^{-2}

^a See footnote to Table 18.2 for key to antibiotics.

The transconjugants obtained were further analysed for their MIC values against gentamicin and the linkage of unselected markers to the gentamicin resistance gene (Table 18.4). It turned out that all strains, except the multi-resistant isolate 6690, transferred their drug resistance *en bloc*. In addition, it was observed that the two strains 8580 and 7446 could transfer either a low

Table 18.4 LINKAGE GROUPS AND MIC VALUES IN GENTAMICIN-RESISTANT TRANSCONJUGANTS^a

Strain	Resistance	MIC (µg/ml)	Selected marker	Phenotype obtained
5316	Gn	128	Gn	Gn (128)
7448	Gn	128	Gn	Gn (128)
8580	Gn, Ap	32	Gn	Gn (32), Ap
			Ap	Gn (8), Ap
6690	Gn, Ap, Tc, Cm	128	Gn or Ap	Gn (32), Ap
			Tc or Cm	Gn (32), Ap, Tc, Cm
7446	Gn, Tc, Cm	32	Tc or Cm or Gn	Gn (4), Cm, Tc

^a See footnote to Table 18.2 for key to antibiotics.

level of gentamicin resistance (MIC of 8 µg/ml or 4 µg/ml) or, at a lower frequency, a high level of resistance (MIC of 32 µg/ml). The transconjugants which had received the low level of resistance could subsequently be converted to the higher level of resistance at frequencies of about 5×10^{-7} . Plasmid DNA isolations from all five transconjugants showed in all cases that a transfer event of the large plasmid (Figure 18.2) had taken place (data not shown).

As mentioned above, the multi-resistant strain 6690 behaved differently in mating experiments. Upon selection for gentamicin or ampicillin resistance, one linkage group exhibiting resistance only against these two drugs was obtained. In contrast, the selection for resistance against tetracycline or chloramphenicol resulted in transconjugants showing the complete multi-resistance. A more detailed analysis of this strain showed a remarkable

repertoire of plasmid interactions and should be discussed on the basis of the data presented in *Figure 18.3*.

The wild-type isolate carried three plasmids, as shown in tracks 1 and 9. They were named pRQ9, pRQ10 and pRQ11 and had molecular weights of 112, 90 and 58 Md. A plasmid curing experiment allowed the isolation of a tetracycline-, chloramphenicol-sensitive derivative which lacked pRQ9. Matings performed at 22°C showed the transfer of the gentamicin and ampicillin resistance at low frequency. The transconjugants obtained carried only pRQ10, as shown in track 3. If these matings were carried out at 37°C, the transfer frequency increased three orders of magnitude and all transconjugants carried, in addition, pRQ11. From these data it was concluded that pRQ9 carries the tetracycline and chloramphenicol resistance genes and that pRQ10 carries the gentamicin and ampicillin resistance genes. pRQ10 is an R-factor which is self-transferable at 22°C and has its transfer optimum at 37°C. At this temperature, it can also mobilize the cryptic plasmid pRQ11.

The analysed transconjugants which had received the complete multi-resistance upon selection for transfer of the tetracycline or chloramphenicol resistance genes revealed the existence of a cointegrate of pRQ9 and pRQ10

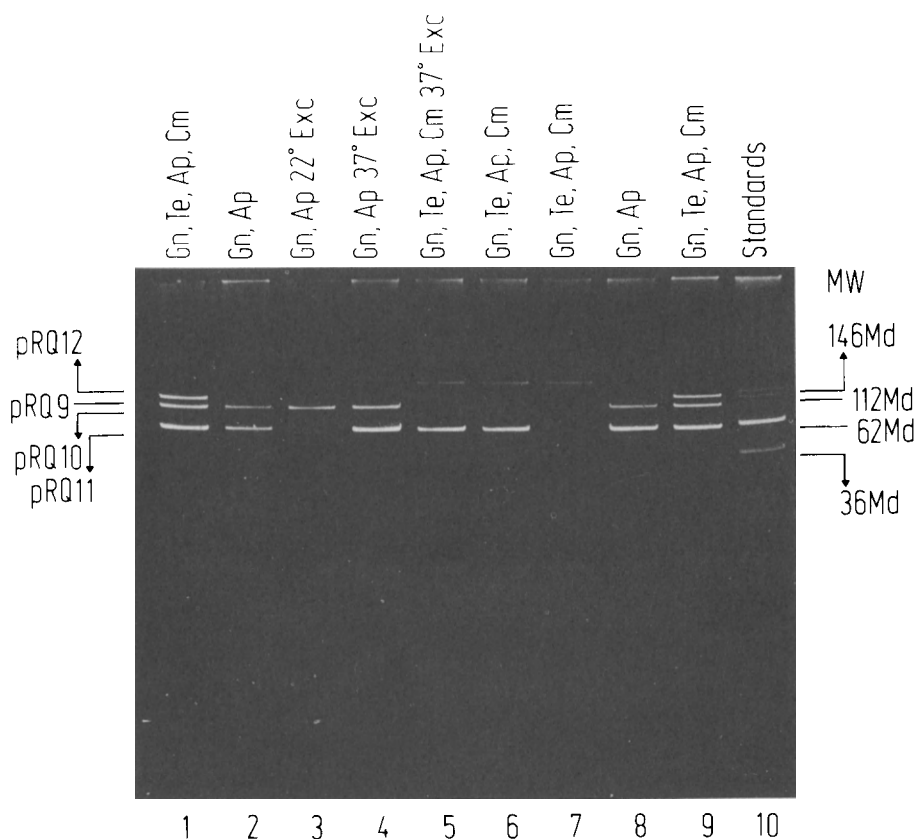


Figure 18.3 Plasmid DNA of the multi-resistant strain 6690 and its transconjugants. Exc = transconjugant

with a molecular weight of 203 Md (track 5). In addition, mobilization of pRQ11 was also observed, because the cointegrate has the transfer characteristics of pRQ10.

If such a multi-resistant transconjugant was used as a donor in a second mating with *Escherichia coli* K-12 at 22°C, two types of transconjugants could be detected. One of the three analysed showed the same plasmids as the donor (track 6), while two of the three carried only the cointegrate (track 7). Obviously, the transfer process at 22°C is not efficient enough to mobilize pRQ11 in all the cases.

If the mating was performed at 37°C with a selection for transfer of the tetracycline or chloramphenicol resistance genes, all ten transconjugants investigated looked like the one analysed in track 6. In contrast to that, we observed that the selection for transfer of gentamicin or ampicillin resistance genes gave rise to transconjugants, as shown in track 8. Two out of seven carried pRQ10 and pRQ11. The existence of these transconjugants can only be explained by the dissociation of the cointegrate.

The data presented here demonstrate the emergence of gentamicin-resistant salmonellae from a turkey farm after prophylactic use of gentamicin. The resistance emerged on two epidemiologically unrelated plasmids; one belonging to *IncH* and one to *IncC*.

The plasmids are self-transferable and the *IncC* plasmid is able to form a plasmid cointegrate. Such R-factor co-integrate formation is now often observed in salmonella strains and seems to play an important role in the formation of multiple resistance.

Gentamicin, like other aminoglycoside antibiotics, is a therapeutically important and widely used bactericidal agent. It is generally used for the treatment of *Pseudomonas aeruginosa* infections and septicaemia induced by Gram-negative organisms. For this reason, the emergence of gentamicin resistance could indeed have serious implications for other areas of medicine, especially when, as could have been predicted in this instance, the prophylactic use of an antibiotic would not lead to the elimination of the pathogenic organism from the environment. Thus, the associated emergence of gentamicin resistance documented here serves to highlight a situation where the administration of an antibiotic can be considered a definite 'malefit' for animal and human medicine.

DIET, DISEASE AND DRUG RESISTANCE IN WILD AND DOMESTICATED BIRDS

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There are two main reservoirs of antibiotic-resistant enteric bacteria. One exists in farm livestock, principally intensively reared pigs and poultry, and has been selected and maintained by mass medication with antibiotics either to prevent or treat disease. The other is in humans; excretion of antibiotic-resistant coliforms occurs throughout the population, but is much higher in the hospital environment where antibiotic usage is concentrated (Richmond, 1972).

Modern agricultural practice has always been based on the most efficient production of food. The introduction of antibiotics into human and veterinary medicine was soon extended to animal production, with the incorporation of sub-therapeutic levels in animal feeds to increase food conversion rates and weight gain. This also resulted in high levels of transferable resistance to therapeutically useful antibiotics in the normal intestinal microflora. Fears that this resistance would be passed to related pathogens like salmonella, and so render many important antibiotics ineffective, led to the setting up of a committee to investigate the problem. The report of the committee, known as the 'Swann Report' (Swann Committee, 1969), recommended that therapeutically useful antibiotics should only be available on prescription. Those used for growth promotion, 'feed antibiotics', should have little or no therapeutic action and should not impair the efficacy of a prescribed antibiotic by selecting for its resistance. The intention was to control the development of resistant organisms in the intestinal microflora of livestock.

In the four years following the implementation of the recommendations, H. W. Smith (1977) monitored the incidence of tetracycline-resistant *Escherichia coli* in pigs, and found little decrease. The Swann Report had little influence on the amount of antibiotics sold for veterinary use (Linton, 1977). The intensification of animal production has made mass prescription of antibiotics commonplace.

Antibiotic resistance in poultry

Animal production is most intensive in the rearing of poultry, and this has increased the risk of disease due to the protozoan infection, coccidiosis. It has

been estimated that in the absence of suitable anti-coccidial drugs, this disease would account for a 20% loss in birds and an increase in production costs of 50–100% (Krylov *et al.*, 1975). The sulphonamide group of antimicrobial agents has been used prophylactically for many years to prevent coccidiosis, and as a consequence the enteric microflora of poultry has been exposed to selective pressures.

Table 19.1 shows a typical antibiotic resistance pattern of coliforms in the faeces of chickens reared in a poultry unit where sulphonamides were one of

Table 19.1 TOTAL AND RESISTANT COLIFORMS
IN 20 FAECAL SAMPLES FROM 6-DAY-OLD
CHICKENS IN INTENSIVE POULTRY UNIT

Total coliforms	Percentage of total coliforms resistant to: ^a			
	C	A	T	S
9.7×10^9	—	—	1.0	1.1
2.3×10^6	—	—	1.4	5.0
3.1×10^5	—	—	24.0	22.0
7.7×10^9	—	—	0.6	1.5
8.3×10^5	—	—	6.9	15.8
2.0×10^7	—	—	60.7	52.0
1.3×10^7	—	—	17.6	20.1
1.1×10^8	—	—	6.2	12.3
5.2×10^5	—	—	2.6	3.5
4.3×10^6	—	—	0.4	1.1
4.0×10^5	—	—	7.2	0.7
1.7×10^6	—	—	2.7	1.8
2.7×10^6	—	—	22.5	18.7
4.1×10^5	—	—	10.0	26.4
1.4×10^6	—	—	30.0	28.0
6.0×10^6	—	—	11.2	11.4
2.4×10^6	—	—	0.4	4.4
2.3×10^6	—	—	3.0	5.9
3.2×10^5	—	—	11.6	11.1
3.5×10^7	—	—	11.7	8.3

^a C, chloramphenicol 25 µg/ml; A, ampicillin 25 µg/ml; T, tetracycline 25 µg/ml; S, streptomycin 25 µg/ml

a number of anti-coccidial drugs used. The high levels of resistance to tetracycline and streptomycin were linked to sulphonamide resistance. A typical tetracycline-resistant colony from each sample was found to be an *E. coli* capable of transferring resistance to tetracycline, streptomycin and sulphonamide to a sensitive *E. coli* recipient.

Although other coccidiostats unrelated to the sulphonamide group are now being used, for example monensin and DNOT, resistance to tetracycline remains high in poultry. This is probably due to medication with the antibiotic and the development of resistant strains able to compete successfully with sensitive ones in this environment.

Animal to human routes of infection

The spread of pathogens like salmonella to the human population via poultry meat has long been known. The spread of antibiotic-resistant *E. coli* from animal to man has been harder to verify.

At the time of the Swann Report it was thought that the *E. coli* serotypes inhabiting the intestines of man and animals differed, and animal strains would not survive long in the human gut. However, Linton *et al.* (1972) showed that healthy adults working with farm animals had a considerably higher incidence of drug-resistant coliform bacteria than their urban counterparts. Howe and Linton (1976) demonstrated that there was considerable overlap in the *E. coli* serotypes carrying transmissible resistance in calves and humans. Colonization of a human gut with an antibiotic-resistant *E. coli* from handling a contaminated chicken carcass was reported by Linton *et al.* (1977).

The major danger has always been the spread of R-factors between the normal intestinal microflora and pathogens like salmonella. There is evidence that this can occur. Aserkoff and Bennett (1969) reported an outbreak of salmonellosis involving 1900 people, in which the causative organism was an antibiotic-sensitive *Salmonella typhimurium*. The faeces of 272 of those affected were examined. All isolates from 87 people who had received no antibiotic treatment were sensitive to antibiotics. The remaining 185, who had received antibiotics, yielded 18 resistant *S. typhimurium*, most of which were shown to contain R-factors, presumably received by *in vivo* transfer from the normal enteric microflora. Conversely, Linton, Timoney and Hinton (1981) reported the probable transfer of an R-factor from a multiple resistant *S. typhimurium* infecting calves to a range of the resident *E. coli* serotypes, where it became established and persisted long after the original infection cleared up. Thus, there does appear to be a route by which resistant coliforms can pass from the livestock reservoir to humans, and the possibility that the R-factors can be passed to related pathogenic species.

Human to animal routes of infection

SEWAGE

The scope for infection to pass from humans to livestock is less obvious. The disposal of sewage onto agricultural land has raised the question of spread of disease to animals (Williams, 1979), and guidelines have been published to prevent this. A 3-week interval before grazing pastures spread with digested sludge, and 6 months for those spread with untreated sludge, is recommended (ADAS, 1982). Sewage is an obvious source of pathogens, especially salmonella. McCoy (1979) noted that the numbers of salmonellae in crude sewage followed a seasonal pattern of distribution closely associated with human infection. The serotypes found in sewage sludges were similar to those infecting the human population.

Jones *et al.* (1981) examined sewage for bacteria of significance to animal health, among them *E. coli*. Of 125 isolates, 23 were haemolytic and 11 belonged to 0-serotypes involved with disease in calves and piglets. However, efficient treatment and disposal reduced the hazard significantly.

BIRDS

A number of bird species have become adapted to feeding on the waste from modern society. Among the most proficient are the gulls, which utilize dumps

and sewage outfalls as major sources of food.

Each summer more than 400 sewage outfalls dispose of sewage from 6 million people in Britain. More than half discharge crude sewage onto the beach or into shallow water. In addition, large cities dispose of crude sewage into estuaries like the Humber, Severn and Mersey (Pearce, 1981).

The availability of these food sources has been a major factor in the increase in the scavenging gull population, with 333 000 breeding pairs of herring gulls (*Larus argentatus*), on coastal sites in Britain, 74 000 of black-headed gulls (*Larus ridibundus*) and 22 000 of great black-backed gulls (*Larus marinus*) in the 1969–70 season (Cramp, Bourne and Saunders, 1974). This does not take into account non-breeding juveniles and the large breeding colonies which now exist inland. The most successful species, the herring gull, has been increasing at the rate of 13% per annum since 1950 (Chabryzk and Coulson, 1976) and must now number over 1 million.

These birds are important agriculturally because after feeding they roost in nearby fields to rest, preen and defaecate (Vernon, 1970). These fields are often grazed by livestock. They have the short grass which gives the birds their instinctive requirement of good all-round vision, to see approaching

Table 19.2 RESISTANCE PATTERNS AND TRANSFERABLE RESISTANCE FROM SALMONELLA SEROTYPES FOUND IN GULL FAECES

Serotypes	Resistance pattern ^a	R-factors
<i>S. typhimurium</i>	CANTS	CANTS
<i>S. typhimurium</i>	NTS	TS
<i>S. typhimurium</i>	ST	ST
<i>S. typhimurium</i> (× 2)	S	S
<i>S. typhimurium</i>	S	—
<i>S. typhimurium</i>	T	T
<i>S. derby</i> (× 5)	T	T
<i>S. give</i> (× 2)	NTS	NTS
<i>S. panama</i> (× 2)	T	T
<i>S. agona</i>	T	T
<i>S. agona</i>	T	—
<i>S. chester</i>	TS	TS
<i>S. hadar</i>	S	S
<i>S. virchow</i>	TS	—

C, chloramphenicol 10 µg; A, ampicillin 10 µg; N, neomycin 10 µg; T, tetracycline 10 µg; S, streptomycin 10 µg—Oxoid Multodisk 3866E.

predators and be seen by their fellows. These pastures become contaminated with faecal organisms, which could be a source of livestock infection.

A review of outbreaks of salmonellosis in livestock from 1978 to 1980 in Scotland described 13 incidents, 11 involving cattle and 2 among sheep, which may have involved gulls (Anon., 1981)

Fenlon (1981), reporting the results of a survey of salmonella serotypes carried by gulls, noted that the range and frequency was similar to that found in the human population, suggesting that sewage was the probable source of gull contamination. The majority of the 160 isolates in this survey were

Table 19.3 THE INCIDENCE OF SALMONELLA AND TOTAL AND ANTIBIOTIC-RESISTANT COLIFORM IN SEWAGE AND IN THE FAECES OF GULLS FEEDING AT THE SEWAGE WORKS

Gull faeces, total coliforms (per g)	Percentage of total coliforms resistant to: ^a				Salmonella
	C	A	T	S	
9.5×10^9	0.003	0.74	1.09	0.93	—
5.2×10^8	0.09	1.08	0.39	0.67	+
1.5×10^8	0.19	2.39	1.40	1.00	+
3.4×10^9	0.18	4.00	2.00	3.29	+
9.3×10^9	0.23	3.47	0.95	2.00	+
1.4×10^6	—	1.13	—	—	—
7.0×10^5	—	3.28	—	—	—
7.1×10^6	—	40.00	9.59	0.20	—
1.9×10^5	16.15	16.15	0.95	15.24	—
2.8×10^7	0.04	1.91	1.44	2.82	—
7.5×10^6	0.77	7.14	1.71	4.86	+
3.8×10^7	0.08	7.99	0.26	2.00	+
2.2×10^8	0.01	3.71	0.26	0.35	+
4.0×10^7	0.11	18.88	0.45	1.89	+
3.6×10^9	0.01	0.79	0.17	0.09	+
2.5×10^5	22.43	24.22	1.21	18.17	—
8.1×10^4	—	—	—	—	+
1.1×10^6	—	1.09	—	—	+
4.1×10^5	—	—	—	—	—
4.1×10^5	—	1.02	—	—	—
% Carriage	65	90	70	70	55
Sewage, total coliforms (per g)	Percentage of total coliforms resistant to:				No. salmonella (per ml)
	C	A	T	S	
3.2×10^6	0.04	27.5	1.13	11.88	2.4×10^1

^a C, chloramphenicol 25 µg/ml; A, ampicillin 25 µg/ml; T, tetracycline 25 µg/ml; S, streptomycin 25 µg/ml.

sensitive to all the antibiotics tested (chloramphenicol, ampicillin, tetracycline, streptomycin, neomycin, furazolidone and sulfamethoxazole/trimethoprim. However, 21 isolates did show resistance to one or more antibiotics, the majority of which were transferable (*Table 19.2*). One isolate of *S. typhimurium* was resistant to at least five antibiotics.

The percentages of antibiotic-resistant coliforms in faecal samples from individual gulls which had been feeding on raw sewage are shown in *Table 19.3*. The proportion of antibiotic-resistant organisms are similar to those found in the sewage, chloramphenicol being lowest, ampicillin highest, with tetracycline and streptomycin intermediate. The high rate of carriage of salmonella by the gulls is also shown, together with the number of salmonellae organisms found in the raw sewage (24/ml).

The proportion of resistant coliforms in sewage is shown in *Table 19.4*. The frequency of transfer was low, and most multiple resistance was linked to the carriage of chloramphenicol resistance, and so usually formed much less than 1% of the total coliform population.

Table 19.4 RESISTANCE AND TRANSFER OF COLIFORMS ISOLATED FROM SEWAGE

Antibiotic	Source of sewage	Percentage of total resistant	Resistance pattern (transferable) ^a			
Chloramphenicol	Sewage works	0.04-0.7	CT	, (-)	; CATS	, (-)
	Harbour outfall		CATS	, (CATS)	. CATS.S × T	, (CATS S × T)
	Sea outfall		CT	, (CT)	; CAT	, (-)
Ampicillin	Sewage works	9.2-27.5	A	, (-)	; A	, (-)
	Harbour outfall		ATS	, (-)	; CAT	, (CAT)
	Sea outfall		CATS	, (CATS)	; A	, (-)
Tetra-cycline	Sewage works	1.1-6.8	TS	, (-)	; TS	, (TS)
	Harbour outfall		CATS	, (CATS)	; CANT.S × T	, (-)
	Sea outfall		ANTS	, (NTS)	; AT	, (-)
Streptomycin	Sewage works	1.1-11.9	AS	, (-)	; ATS	, (-)
	Harbour outfall		S	, (-)	; AS	, (-)
	Sea outfall		ATS	, (-)	; AS	, (-)

^a C, chloramphenicol; A, ampicillin; N, neomycin; T, tetracycline; S, streptomycin; S × T, sulfa-methoxazole/trimethoprim. Underlined = resistance carried by *Escherichia coli*.

Many of the coliforms carrying ampicillin resistance were not *E. coli*. This resistance was often not linked to resistance to any other antibiotic, and was not transferable. Some of these isolates were identified as *Klebsiella* and *Enterobacter* species. These species accounted for much of the high levels of ampicillin resistance found in both gulls and sewage.

A similar frequency and pattern of resistance were found in gull isolates, especially those from birds feeding at sewage outfalls (Tables 19.5 and 19.6). Table 19.7 shows isolates from faeces of gulls resting in an urban park area, where the level of carriage of chloramphenicol resistance was much lower. However, as in sewage, chloramphenicol-resistant isolates from gull faeces tended to carry multiple resistance. A common pattern is CANTS, which is frequently encountered in *Salmonella typhimurium* phage type 193 in cattle (Sojka and Wray, 1980).

The type of ampicillin resistance found in gulls was similar to that in sewage, often carried by coliforms other than *E. coli*, not linked to other resistance and not transferable.

The most frequently encountered multiple transferable resistance in gulls was ampicillin and streptomycin which was often carried by 1% of the total coliforms.

Many of the resistant coliforms found in gulls faeces and sewage were species other than *E. coli*. Isolates from the same source on one occasion would be predominantly *E. coli* and on the next be other coliform species. Whereas *E. coli* isolates may give higher frequencies of transfer to an *E. coli* recipient, the other coliform species can and do carry and transfer multiple antibiotic resistance and so may be a significant source of R-factors.

Table 19.5 RESISTANCE AND TRANSFER PATTERNS OF COLIFORMS FROM FAECES OF GULLS FEEDING AT AN UNTREATED SEWAGE OUTFALL

<i>Antibiotic</i>	<i>Carriage (%)</i>	<i>Percent- age of total resistant</i>	<i>Resistance pattern (transferable)^a</i>			
Chloram- phenicol	85.7	0.01- 2.78	CATS	, (-)	; CANTS	, (CT)
			CANTS	, (CATS)	; CANTS.S × T	, (CATS.S × T)
			CANTS	, (CANTS)	; CANTS.S × T	, (-)
Ampicillin	100	2.5-36.4	A	, (-)	; AS	, (-)
			A	, (-)	; CATS	, (-)
			A	, (-)	; CANTS.S × T	, (CATS.S × T)
			A	, (-)	;	
Tetracycline	85.7	0.27- 3.15	CATS	, (-)	; CANTS.S × T	, (-)
			I	, (T)	; CANTS.S × T	, (CATS.S × T)
			TS	, (-)	;	
			TS	, (-)	;	
Streptomycin	100	0.9-18.2	AS	, (AS)	; S	, (-)
			AS	, (-)	; S	, (-)
			CATS	, (-)	; S	, (-)
					S	, (-)

^a See footnote to Table 19.4 for key.**Table 19.6** RESISTANCE AND TRANSFER PATTERNS OF COLIFORMS FROM FAECES OF GULLS FEEDING AT A SEWAGE WORKS

<i>Antibiotic</i>	<i>Carriage (%)</i>	<i>Percentage of total resistant</i>	<i>Resistance pattern (transferable)^a</i>			
Chloram- phenicol	60.0	0.04-22.4	CT	, (-)	; CATS	, (-)
			CAS	, (S)	; CATS	, (-)
			CAT	, (-)	; CANTS	, (-)
Ampicillin	100	1.08-40.0	A	, (-)	; AS	, (-)
			A	, (-)	; AS	, (-)
			A	, (-)	; ANS	, (-)
			A	, (-)	; ATS.S × T	, (-)
			A	, (-)	; CAS	, (CS)
Tetracycline	70.0	0.26-9.59	T	, (-)	; TS	, (-)
			T	, (-)	; ATS	, (-)
			TS	,	; CATS.S × T	, (TS)
			TS	, (-)	;	
Strepto- mycin	70.0	0.20-18.17	S	, (-)	; AS	, (-)
			AS	, (-)	; TS	, (-)
			AS	, (AS)	; CAS	, (-)
			AS	, (S)	;	

^a See footnote to Table 19.4 for key.

Table 19.7 RESISTANCE AND TRANSFER PATTERNS OF COLIFORMS FROM FAECES OF GULLS IN AN URBAN AREA

<i>Antibiotic</i>	<i>Carriage (%)</i>	<i>Percentage of total resistant</i>	<i>Resistance pattern (transferable)^a</i>			
Chloramphenicol	10.0	0.004	CANTS (CANTS)			
Ampicillin	100	3.0-16.9	A	, (-)	; AS	, (A)
			A	, (-)	; AS	, (AS)
			A	, (-)	; AS	, (AS)
			ATS	, (-)	; AS	, (AS)
			ATS	, (-)	; ATS	, (AS)
Tetracycline	70.0	0.1-17.3	CANTS	, (-)	; TS	, (-)
			TS	, (-)	; TS	, (TS)
			ATSFR	, (-)	; ATS	, (TS)
Streptomycin	60.0	0.2-4.0	S	, (-)	; ATS	, (AS)
			AS	, (AS)	; ATS	, (-)
			ATS	, (AS)	; ATS	, (-)

^a See footnote to Table 19.4 for key.

Apart from pastures, gulls congregate in areas where fresh water is available. Here they wash and, on large bodies of water, roost overnight. Lakes and reservoirs are particularly favourite roosts, and Fennell, Jones and Morris (1974) found in excess of 1800 *E. coli*/100 ml and up to 15 salmonellae/l in water from a reservoir frequented by gulls.

The use of such water, untreated on the farm, can have serious consequences. Johnston, MacLachlan and Hopkins (1979) recorded a series of outbreaks of salmonellosis on a dairy farm which used a nearby loch as a source of drinking water for its cows. The loch was frequented by gulls which fed at a local town's sewage outfall. The outbreaks ceased when mains water was installed.

While it is reasonably well established that salmonella carried by birds can cause livestock infection, it does not appear that the numbers present in the birds' faeces are high. Fennell, Jones and Morris (1974) concluded that gulls roosting at a reservoir were only vectors as their salmonellae numbers were too low to count by direct plating. Fenlon (1981) found only 0.18-191 salmonellae/g in a number of faecal samples examined. This suggests that, in many instances, gulls might be just carrying the organism and not be actually infected. Outbreaks of salmonellosis do occur in gull colonies in the breeding season, when the birds are crowded together and under stress. However, livestock infections in which gulls have been implicated have occurred throughout the year, suggesting that the cattle and sheep do become infected by the ingestion of small numbers of salmonellae.

Colonization of livestock by *Escherichia coli* and salmonella

It is significant that it is cattle and sheep which are most at risk through contamination of pastures and water supplies by birds. These two species

have lower levels of resistant coliforms in their gut microflora as their production has not involved the same degree of intensification as pigs and poultry.

Most work published shows that high numbers of salmonellae ($> 10^6$ organisms) are required to cause disease, although lower numbers can give symptomless excretion. Certain conditions, for example alteration of the normal microflora by antibiotic treatment, can predispose animals to infection by significantly lower dose rates (Lee, 1978). Work on the effects of food intake on the establishment of *E. coli* and salmonella in the bovine rumen showed that these organisms were readily eliminated by animals feeding regularly and well. A decrease or interruption in food supply allowed salmonella and *E. coli* to multiply; also, starvation for 2–3 days resulted in infection of the intestine (Brownlie and Grau, 1967).

Smith (1975) demonstrated that starving sheep for 24–48 h enabled a mixed inoculum of 10^3 donor and recipient *E. coli* to become established in the rumen and transfer of R-factors to take place. The recipient of R-factors survived at least 6 weeks. In healthy non-starved animals, doses of 10^{10} *E. coli* failed to become established.

The same author (M.G. Smith, 1977) showed that starvation of sheep for 24–48 h enabled low doses (4.4×10^2) of *S. typhimurium* to infect and cause death in a sheep. At the same time, an antibiotic-resistant *E. coli* was also able to increase from an initial level of 10^2 – 10^4 and transfer resistance (in the absence of selective pressure from antibiotic treatment) to the infecting *S. typhimurium*. At the time of death, the sheep was excreting 10^9 salmonellae/g of faeces, 10^6 containing the transferred R-factor.

It would appear that the conditions which enable salmonella infections to become established in ruminants may be similar to those required for antibiotic-resistant *E. coli* establishment. These conditions also appear to be suitable for R-factor transfer.

Antibiotic resistance patterns as an indicator of feeding habits

The antibiotic resistance pattern of faecal coliforms of intensively reared poultry shows a characteristic high level of resistance to tetracycline and streptomycin (Table 19.1), with a low or non-existent resistance to chloramphenicol and ampicillin. Coliforms in the faeces of gulls feeding at sewage outfalls show a similar resistance pattern to those found in the sewage. Fenlon (1981) described how the incidence of salmonella in gulls was highest in those near sources of sewage and decreased as gulls became less reliant on it as a food source. Table 19.3 shows a typical resistance pattern of coliforms from faeces of gulls scavenging on sewage; it also shows an incidence of 55% of salmonella in these gulls.

A similar decreasing trend to that found in salmonella can be found in resistant coliforms when the faecal microflora gulls from urban and rural habitats are compared (Figure 19.1).

That this lack of antibiotic-resistant coliforms in rural gulls is due to different feeding habits can be supported by examining other birds which feed or congregate on pastures, but do not exploit sewage as a food source. Faecal samples from a large number of rooks, feral pigeons and wild geese were

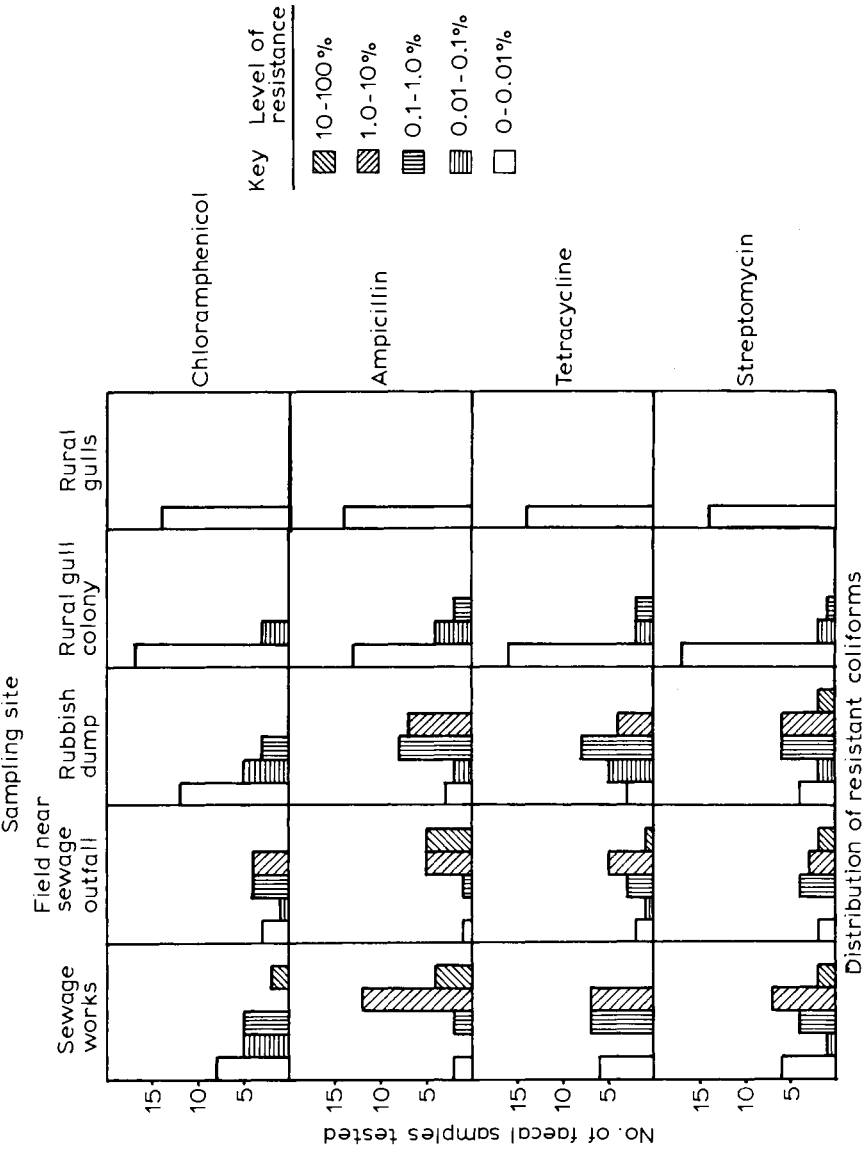


Figure 19.1 Histogram showing relationship between the availability of sewage and urban waste and the incidence of antibiotic resistance in coliforms in rural and urban gulls

examined. Like rural gulls, all showed an absence or very low level of antibiotic-resistant coliforms and all rural gulls and other bird species studied had no salmonella in their faeces.

The pattern of antibiotic resistance can therefore be used as a simply performed and reliable guide to the feeding habits of the bird species involved, and the possible presence of the faecal pathogen salmonella. It does not, however, serve as an indication of the other important faecal organism *Campylobacter*, which was found frequently in all bird species studied.

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THE CONTROL OF BACTERIAL FISH DISEASES BY ANTIMICROBIAL COMPOUNDS*

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Introduction

In the fish farm environment where stocking intensities are high, poor hygiene may develop and disease may ensue (McCarthy, 1980). Although there is no general agreement on the definition of disease, most individuals will be familiar with the concept, and an appropriate formulation might be as defined by Campbell, Scadding and Roberts (1979):

A disease is the sum of the abnormal phenomena displayed by a group of living organisms in association with a specified common characteristic or set of characteristics by which they differ from the norm of their species in such a way as to place them at a biological disadvantage. . . .

Thus, it may be inferred that 'disease' is heterogeneous, describing many abnormal conditions in an individual of a species.

To date, 19 bacterial genera have been implicated as pathogens of fish (Table 20.1). In the UK, *Aeromonas salmonicida*, *Flexibacter columnaris* and *Renibacterium salmoninarum* are the causal agents of furunculosis, columnaris and bacterial kidney disease, respectively, which are notifiable diseases under the Diseases of Fish Act, 1937. The presence of pathogens in fish is undesirable, and consequently much effort is made to rid stocks of disease. Methods for disease control include good management practices and the use of vaccines for prophylaxis and antimicrobial compounds (drugs) for treatment. The latter will be discussed further.

Bacterial diseases of fish

Fish diseases attributed to bacteria have been included in Table 20.1. Information about acinetobacter disease, flavobacteriosis, lactobacillosis and pasteurellosis is incomplete and these will not be considered further.

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Table 20.1 BACTERIAL DISEASES OF FISH

<i>Bacterial pathogens</i>	<i>Disease</i>	<i>Host range</i>	<i>Geographical distribution</i>
<i>Acinetobacter</i> sp.	Acinetobacter disease	Channel catfish (eggs)	USA
<i>Aeromonas hydrophila</i> (<i>A. liquefaciens</i> , <i>A. punctata</i>).	Haemorrhagic septicaemia (motile aeromonas septicaemia), redsore disease	Most freshwater fish	Worldwide
<i>A. salmonicida</i> (<i>Haemophilus piscium</i>)	Furunculosis (carp erythrodermatitis) (ulcer disease)	Bass Many freshwater fish species	USA Worldwide
<i>Aeromonas</i> sp.	Fin rot	Salmonid fish	Worldwide
<i>Clostridium botulinum</i>	Botulism	Salmonid fish	Denmark, England
<i>Cytophaga psychrophila</i>	Coldwater disease	Salmonid fish	Australia, USA
<i>Edwardsiella ictalura</i>	Enteric septicaemia of catfish	Catfish	USA
<i>E. tarda</i> (<i>Paracolobactrum anguillimortiferum</i>)	Red pest, edwardsiellosis, emphysematous putrefactive disease of catfish	Eels, channel catfish	Japan, USA
<i>Eubacterium tarantellus</i>	—	Channel catfish Striped mullet	USA USA
<i>Flexibacter</i> sp.	Fin rot	Salmonid fish	Worldwide
<i>F. columnaris</i>	Columnaris (saddleback disease)	Most freshwater fish species	Worldwide
<i>Lactobacillus</i> sp.	Lactobacillosis	Salmonid fish	North America
<i>Mycobacterium</i> spp. (<i>M. anabanti</i> , <i>M. fortuitum</i> , <i>M. marinum</i> , <i>M. piscium</i> , <i>M. platyaequalis</i> , <i>M. ranae</i> , <i>M. salmoniphilum</i>)	Mycobacteriosis	Most fish species	Worldwide
<i>Myxobacter</i> sp.	Bacterial gill disease	Most fish species	Worldwide
<i>Nocardia asteroides</i> (<i>N. kansasii</i>)	Nocardiosis	Most fish species	Worldwide
<i>Pasteurella piscicida</i>	Pasteurellosis, pseudotuberculosis	White perch, striped bass, yellowtail	USA Japan
<i>Pseudomonas</i> sp.	Fin rot	Mostly salmonid fish	Worldwide
<i>P. anguilliseptica</i>	Red spot	Eels	Japan, Scotland
<i>P. fluorescens</i>	Generalized septicaemia	Most fish species	Worldwide
<i>Renibacterium salmoninarum</i>	Bacterial kidney disease, Dee disease	Salmonid fish	Europe, Japan, North America
<i>Serratia liquefaciens</i>	Salmonid blood spot	Brook trout, Atlantic salmon	Australia
<i>Yersinia enterocolitica</i> / <i>Y. ruckeri</i>	—	Immature salmonid fish	Scotland, USA
<i>Sporocytophaga</i> sp.	Streptococcosis	Many fish species	Japan, South Africa, USA
<i>Streptococcus</i> spp. (<i>S. faecium</i> , <i>S. faecalis</i> , <i>S. agalactiae</i>)	—	—	—
<i>Vibrio alginolyticus</i>	Septicaemia	Sea bream	Israel
<i>V. anguillarum</i>	Vibriosis	Most marine fish species	Worldwide
<i>V. damsela</i>	Vibriosis	Damsel fish	USA
<i>V. ordalii</i> (formerly <i>V. anguillarum</i> biotype II)	Vibriosis	Marine fish species	Extensive
<i>V. parahaemolyticus</i>	Vibriosis	Marine fish species	—
<i>V. cholera</i> (non-01)	Vibriosis	Ayu	Japan
<i>V. vulnificus</i>	Vibriosis	Eels	Japan, North America
<i>Yersinia ruckeri</i>	Enteric redmouth	Salmonid fish	Australia, Italy, England, North America

BACTERIAL KIDNEY DISEASE

This is a severe debilitating disease of salmonid fish, notably in Europe, Japan and North America. It is a chronic, systemic infection, for which the external signs include exophthalmia, distended abdomen and blood-filled blisters on the flank. The heart, kidney, liver and spleen may contain local foci of infection. The disease has high mortality rates, and is difficult to treat. The aetiological agent is a Gram-positive coccobacillary organism, classified in a new genus as *Renibacterium salmoninarum* (Fryer and Sanders, 1981).

BOTULISM

This has been reported in rainbow trout collected in Denmark (Huss and Eskildsen, 1974) and England (Cann and Taylor, 1982). Characteristic disease symptoms are vague, although infected fish display erratic swimming until death ensues. *Clostridium botulinum* type E, an anaerobic, Gram-positive spore-forming organism, is responsible for the condition.

CARP ERYTHRODERMATITIS

This is a sub-acute to chronic disease of the skin, which occurs at all water temperatures. Infection may start at the site of injury to the epidermis and develop into a haemorrhagic inflammation. This may lead to ulceration and ultimately to generalized oedema. The disease is caused by *A. salmonicida* (Bootsma, Fijan and Blommaert, 1977).

COLDWATER PEDUNCLE DISEASE

This is a septicaemic condition of salmonid fish, which is problematic when water temperatures are about 12°C, i.e. during early Spring (Pacha, 1968). First recognized in 1948, the disease is now widespread in hatcheries of the Pacific North-west, USA. The aetiological agent, *Cytophaga psychrophila*, is homogeneous, although controversy surrounds the validity of the taxon in so far as Lewin and Lounsbery (1969) consider that the organisms belong in the genus *Flexibacter*, as *F. aurantiacus*.

COLUMNARIS

This occurs in most freshwater and, probably, some farmed marine fish species. External lesions appear on the skin, fins and gills. The development of a grey 'saddle' near the dorsal fin may occur. The aetiological agent is a fairly unreactive organism, which exhibits gliding motility, and has been classified as *F. columnaris* (McCarthy, 1975b).

EDWARDSIELLOSIS

This is a septicæmic disease of channel catfish, eels ('red-pest'), cyprinids, large mouth bass, tilapia, mullet and sea-bream (Meyer and Bullock, 1973; Aoki and Kitao, 1981). In catfish, symptoms include haemorrhaging on the head and flank, and, in acute cases (known as emphysematous putrefactive disease), abscesses give rise to gas-filled cavities which if broken emit a foul odour. The causal agent of these conditions is *Edwardsiella tarda*. A newly described species, *E. ictalura*, has been associated with enteric septicæmia of catfish (Hawke, 1979). With this condition, external signs of disease are often absent in fish larger than 15 cm, but, when they do occur, include exophthalmia and lesions on the lateral body surface and in the mouth and throat.

ENTERIC REDMOUTH

This is a haemorrhagic septicæmic disease of freshwater salmonids (Rucker, 1966). Typically, subcutaneous haemorrhages develop along the base of the fin, and in and around the mouth and operculum. Exophthalmia and the erosion of the lower jaw producing reddish ulceration may occur (Busch, 1978). Although initially recognized in Idaho, USA, the disease may be spreading in the USA due to introduction of sub-clinically infected fish and eggs (Wobeser, 1973). The aetiological agent has been classified in a new species of *Yersinia*, as *Y. ruckeri*.

FIN ROT

Poor nutrition, mechanical damage and chemical pollution have been implicated with the occurrence of fin rot in salmonid fish (Bullock and Snieszko, 1970; Ziskowski and Murchelano, 1975). Schneider and Nicholson (1980) reported that the occurrence and severity of fin rot was dependent on the time of year and water temperature. Thus, the condition was most advanced during winter when water temperatures were low. Unidentified *Aeromonas*, *Flexibacter* and *Pseudomonas* spp. have been implicated in the disease.

FURUNCULOSIS

This is a septicæmic disease of a highly communicable nature, which was first described in hatchery salmonid fish in Germany (Emmerich and Weibel, 1894). The 'acute' form of the disease is characterized by the presence of few or no external symptoms, is of sudden onset and causes high mortality. The second, 'sub-acute', type is characterized by the appearance of prominent focal muscle lesions (furuncles), is of more gradual onset and causes lower mortalities (McCarthy, 1980). The aetiological agent is *A. salmonicida*, which is a Gram-negative, non-motile fermentative organism, producing a brown diffusible pigment on tryptone soya agar (Schubert, 1974). An atypical form of the organism, known as *Haemophilus piscium*, is responsible for ulcer disease in goldfish.

GILL DISEASE

This affects warm-water pond fish and salmonids. Symptoms include loss of appetite, sluggishness and crowding near the water surface. The gills appear red, with swollen lamellae (Ashburner, 1978). Gram-negative, slime-producing bacteria, loosely associated with the genus *Flexibacter*, have been implicated as the aetiological agent.

HAEMORRHAGIC SEPTICAEMIA

This is caused by *A. hydrophila*. Acute and fatal septicaemias develop which may be accompanied by external abscesses, ulcers, exophthalmia and abdominal distensions (Eurell, Lewis and Grumbles, 1978).

MYCOBACTERIOSIS AND NOCARDIOSIS

These diseases may be confused with each other. Mycobacteriosis is a chronic, progressive disease which affects > 150 species of freshwater and marine fish (Nigrelli and Vogel, 1963). Various external symptoms develop, including emaciation, inflammation of the skin, exophthalmia, open lesions and ulceration. Internally, greyish white nodules (granulomas) develop on various organs, notably the liver, kidney and spleen. The disease is probably transmitted by ingestion of contaminated food, fish tissue or debris (Dulin, 1979). Various *Mycobacterium* spp. have been implicated with the condition. Similar symptoms occur with nocardiosis, which is caused by *Nocardia asteroides*, primarily.

RED SPOT OF EELS

This was first described in Japan during 1971 (Nakai and Muroga, 1979). Haemorrhagic spots develop on the head and along the fins. The aetiological agent is *Pseudomonas anguilliseptica*.

SALMONID BLOOD SPOT

This has been reported only in Australia (Llewellyn, 1980). Typically, the fish darkened in colour and developed blood spots in the eyes. Abdominal distensions were recorded. The taxonomic position of the pathogen is *sub judice*, although similarities exist to *Yersinia ruckeri*.

STREPTOCOCCOSIS

This is a haemorrhagic disease of a diverse range of marine and freshwater fish (Robinson and Meyer, 1966; Boomker *et al.*, 1979; Kitao, Aoki and Sakoh, 1981). External signs include abnormal pigmentation, exophthalmia

and haemorrhages in the eye chamber. The gross pathology matches the description for haemorrhagic septicaemia. Several poorly described species of *Streptococcus* have been implicated as causal agents.

VIBRIOSIS

This septicaemic disease of marine fish is of mixed aetiology. Historically, marine vibrios, implicated as fish pathogens, have been labelled as *Vibrio anguillarum* (Evelyn, 1971). However, the taxon is heterogeneous, with bio-type II now recognized as a separate species, *V. ordalii* (Schiewe, Trust and Crosa, 1981).

Antimicrobial compounds used for the control of bacterial fish diseases

A diverse range of drugs have found use on fish farms for the control of disease (Snieszko, 1978; Herwig, 1979). These include antibiotics, e.g. chloramphenicol, some of which are important in human medicine, and synthetic compounds, e.g. oxolinic acid, used almost exclusively in fisheries (Table 20.2). It is controversial whether or not medically important compounds should be used for fish, because bacterial resistance may develop and spread rapidly to other bacterial species in the aquatic environment. This problem may be largely resolved by careful licensing and control measures for fisheries compounds. Certainly, stringent precautions are necessary to prevent misuse of drugs which might reduce their value for other potential users. In this respect, many drugs are obtainable only from qualified veterinarians, who have responsibility for their use. Moreover, with some compounds, such as oxytetracycline, the usage must be discontinued for a set period before the fish may be sold for human consumption. This withdrawal period ensures that there are no drug residues left in the fish tissues.

Administration of the antimicrobial compounds

There are four approaches for administering drugs to fish. These include the oral route via medicated food, bath and flush treatments, and by injection. In the first case, drugs may be mixed with normal food and fed to the fish. Usually, the regime leads to the administration of a unit weight of drug to a set weight of fish on a daily basis for a predetermined number of days. For example, the recommended dosage for oxolinic acid is 10 mg of drug/kg of fish/day for 10 days (Austin, Rayment and Alderman, 1983). This method is advantageous in so far as the quantities of drug fed to the fish are carefully controlled, and, if realistic feeding regimes are adopted, only a negligible quantity would enter the aquatic environment. It is important, however, that the drug is palatable to fish and will be absorbed intact through the gut.

Bath treatments, such as with nitrofurantoin (Table 20.3), are necessary if the fish refuse to eat and, therefore, would not consume medicated food. With this method, the fish are placed in a solution or suspension of the drug for a predetermined time. This may be for a few seconds ('dip') or several

Table 20.2 ANTIMICROBIAL COMPOUNDS IN USE FOR CONTROLLING BACTERIAL FISH DISEASES

<i>Bacterial disease</i>	<i>Antimicrobial compounds used</i>
Acinetobacter disease	Iodophors
Bacterial kidney disease	Erythromycin, iodophors, penicillin G, sulfoxazole, sulphamethazine-sodium salt
Botulism	—
Carp erythrodermatitis	Chloramphenicol, furazolidone, oxytetracycline, tetracycline
Coldwater disease	Aureomycin, chlortetracycline, diquat, furanace, oxytetracycline, quaternary ammonium compounds, sulfoxazole, sulphamerazine, sulphamethazine-sodium salt
Columnaris	Acriflavin-neutral, aureomycin, diquat, furanace, malachite green, oxolinic acid, oxytetracycline, tetracycline, quaternary ammonium compounds, sulfoxazole, sulphamerazine, sulphamethazine-sodium salt
Edwardsiellosis	Oxytetracycline
Enteric redmouth	Chloramphenicol, oxolinic acid, oxytetracycline, potentiated sulphamide, sulphamerazine, Tiamulin
Enteric septicaemia of catfish	Oxytetracycline
Emphysematous putrefactive disease of catfish	Oxytetracycline
Fin rot	Albucid, aquarol, benzalkonium chloride, chloramphenicol, copper sulphate, furanace, globucid, kanamycin, malachite green, oxytetracycline, phenoxethol
Flavobacteriosis	Iodophors
Furunculosis	Chloramphenicol, flumequine, Fosfomicina, furazolidone, iodophors, nifurprazine, oxolinic acid, oxytetracycline, potentiated sulphamide, sulfoxazole, sulphamerazine, sulphamethazine-sodium salt, tetracycline
Gill disease	Benzalkonium chloride, diquat, furanace, malachite green, oxytetracycline, quaternary ammonium compounds
Haemorrhagic septicaemia/redsore	Chloramphenicol, furanace, iodophors, kanamycin, oxolinic acid, oxytetracycline, streptomycin, sulphamerazine
Lactobacillosis	—
Mycobacteriosis	Chloramine B or T, cycloserine, doxycycline, ethionamide, isoniazid, kanamycin, minocycline, rifampicin, streptomycin, sulfoxazole
Nocardiosis	Sulphonamides
Pasteurellosis (pseudo-tuberculosis)	Chloramphenicol
Redspot	—
Salmonid blood spot	Methylene blue/oxytetracycline
Streptococcosis	Ampicillin, erythromycin, oxytetracycline, sodium nifurstyrenate, tetracycline
Ulcer disease	Chloramphenicol, oxytetracycline
Vibriosis	Aureomycin, chloramphenicol, 5,7-dichloro-8-hydroxyquinoline, Halquinol, furanace, kanamycin, nifurprazine, nitrofurantoin, oxolinic acid, oxytetracycline, sulfoxazole, sulphamerazine, sulphamethazine-sodium salt

hours. After treatment, the fish are returned to the holding area. It is essential that the compounds are soluble or, if insoluble, are dispersed evenly in the water by use of surfactants (Austin, Morgan and Alderman, 1981). However, a major drawback concerns adequate disposal of the spent drug.

Flush treatments also involve the addition of drug, but in high concentrations, to water although in this case it may occur in the stock tank/pond rather than in a separate bath. After addition, the drug is flushed through the

Table 20.3 METHODS OF ADMINISTERING THE ANTIMICROBIAL COMPOUNDS

<i>Antimicrobial compound</i>	<i>Methods of administration</i>
Acriflavine, neutral	5–10 mg/l in water for several hours to several days
Albucid, sodium salt	100 mg/l in water for 8 h
Ampicillin	200 mg/l in water for undetermined duration
Aquarol	80 mg/l in water as dip, at 3-day intervals
Aureomycin	(a) 10–20 mg/kg of food (b) 10–20 mg/l of water, as a bath
Bacitracin	250–300 mg/gal of water for undetermined duration
Benzalkonium chloride	1–2 mg/l of water for 1 h
Chloramine B or T	18–20 mg/l of water at pH 7.5–8.0; treat for 2–3 days
Chloramphenicol	(a) 50–75 mg/kg of food/day for 5–10 days (b) 10–50 mg/l of water as a bath
Chlortetracycline	(a) 10–20 mg/kg of food; feed normally for 1–4 days (b) 10–20 mg/l of water, as a bath
Cycloserine	30 mg/kg of food
Diquat	2–4 mg/l of water for 1 h
Doxycycline	500 mg/kg of food
Erythromycin	25–100 mg/kg of fish/day for 4–21 days
Ethionamide	Undetermined dosage
Flumequine	6 mg/kg of fish/day for 6 days
Fosfomicina	Unknown dosage
Furanace	(a) 2–4 mg/kg of fish/day for 3–5 days (b) 0.5–1 mg/l of water for 5–10 min, as a bath
Furazolidone	25–75 mg/kg of fish/day for up to 20 days
Globucid	100 mg/l of water for 8 h
Halquinol (5,7-dichloro-8-hydroxy-quinoline)	(a) 25 mg/kg of fish/day for 10 days (b) 25 mg/l of seawater/15 min for 3 days
Iodophors	50–200 mg of available iodine/l of water for 10–15 min
Isoniazid	40 mg/gallon of water
Kanamycin	50 mg/kg of fish/day for 7 days
Malachite green	1–5 mg/l of water for 1 h
Methylene blue	1–3 mg/l of water for 3–5 days
Minocycline	500 mg/kg of food
Nifurprazine hydrochloride	(a) 10 mg/kg of food, fed for 3–6 days (b) 0.01–0.1 mg/l of water, as a bath for an undefined period
Nitrofurantoin	50 mg/l of water, as a bath for 1 h
Oxolinic acid	(a) 10 mg/kg of fish/day for 10 days (b) 1 mg/l of water, as a bath for 24 h (recommended for control of columnaris)
Oxytetracycline	50–75 mg/kg of fish/day for 10 days
Penicillin G	Incomplete information
Phenoxethol	100–200 mg/l of water for 12 h
Quaternary ammonium compounds	1–2 mg/l of water for 1 h
Rifampicin	60 mg/kg of food
Sodium nifurstyrenate	50 mg/kg of fish/day for 3–5 days
Streptomycin	50–75 mg/kg of fish/day for 5–10 days
Sulfisoxazole	200 mg/kg of fish/day for 14 days
Sulphamerazine	200 mg/kg of fish/day for 14 days
Sulphamethazine, sodium salt	100–200 mg/kg of fish/day for 10–20 days
Tetracycline	50–100 mg/kg of fish/day for 10–14 days
Tiamulin	5 mg/kg of fish/day for 14 days
Tribissen/methanol	30 mg/kg of fish/day for 5–7 days

system by normal water flow. This inevitably results in a brief exposure to the compound and, as before, adequate disposal may pose problems.

Injection of solutions containing antimicrobial compounds is feasible for valuable fish, such as brood stock and salmon smolts. However, the technique is slow and usually requires the fish to be anaesthetized prior to injection.

Chemoprophylaxis versus chemotherapy

Drugs may be used for the prevention (chemoprophylaxis) or treatment (chemotherapy) of fish diseases. The former is particularly effective when the occurrence of disease is predictable. For example, at one site in England used for trials with oxolinic acid, furunculosis appeared annually in rainbow trout when the water temperature reached 18°C, usually during July or August (Austin, Rayment and Alderman, 1983). Losses from disease were negligible if the fish received medicated diet, commencing when the water temperature reached 17.5°C.

To be effective, chemotherapy must start as soon as possible after disease occurs. This means that rapid diagnosis and the determination of correct antimicrobial susceptibility profiles are essential. Unfortunately some diseases, such as mycobacteriosis, are extremely difficult to treat, and involve the prolonged use of expensive drugs (Dulin, 1979). In this situation, treatment of large numbers of fish is impractical. Clearly, the maxim is that the cost of the drug should not exceed the value of the lost fish (Snieszko, 1978). The only exception is for small numbers of pet fish.

Suggested control regimes

Information about the control of acinetobacter disease, flavobacteriosis and pasteurellosis is incomplete, whereas for botulism, lactobacillosis and red spot it is negligible. Nevertheless, control of the other bacterial diseases has been well documented, and a list of the drugs used has been included in *Table 20.2*.

BACTERIAL KIDNEY DISEASE

It has been stated that bacterial kidney disease is the most difficult of the bacterial fish pathogens to control (Fryer and Sanders, 1981). Wolf and Dunbar (1959) concluded, after a study of 34 compounds, that erythromycin fed at a rate of 100 mg/kg of fish/day for 21 days achieved the best result.

CARP ERYTHRODERMATITIS

Chloramphenicol, furazolidone, oxytetracycline and tetracycline have been used with some success (Bootsma, Fijan and Blommaert, 1977).

COLDWATER DISEASE

The use of furanace (0.5 mg/l, as a bath for 1 h on every third day, to fry, followed by twice weekly treatment to the fingerlings) has been advocated (Holt, Conrad and Fryer, 1975).

COLUMNARIS

Although sulphonamides have been used for treatment, they are considered to be generally ineffective. Tetracycline, fed at 100 mg/kg of fish/day is more effective if used prophylactically at times of anticipated stress (McCarthy, 1975b).

EDWARDSIELLOSIS

Oxytetracycline, at 55 mg/kg of fish/day, has been reported to improve the condition in 4–7 days (Hawke, 1979).

ENTERIC REDMOUTH

Treatment with sulphamerazine, at 200 mg/kg of fish/day for 5 days, followed by oxytetracycline at 50 mg/kg fish/day for 3 days, has been successful (Klontz and Huddleston, 1976). However, more recently, use of tiamulin (5 mg/kg of fish/day for 14 days), Tribissen (Burroughs Wellcome) (1 mg/kg of fish/day for 14 days) and oxolinic acid (10 mg/kg of fish/day for 10 days) have been recommended (Bosse and Post, 1983; Rogers and Austin, 1983).

FIN ROT AND GILL DISEASE

Bath treatments involving use of benzalkonium chloride, furanace, malachite green and oxytetracycline have been used successfully for these conditions (*Tables 20.2 and 20.3*).

FURUNCULOSIS, ULCER DISEASE AND HAEMORRHAGIC SEPTICAEMIA

Oral treatment with sulphamerazine (200 mg/kg of fish/day for 10–14 days), tetracycline (75 mg/kg of fish/day for 10–14 days), furazolidone (75 mg/kg of fish/day for 10–14 days) and potentiated sulphonamide, e.g. Tribissen (30 mg/kg of fish/day for 5–7 days), have been used successfully to treat the disease (McCarthy, Stevenson and Salsbury, 1974; McCarthy, 1975a). More recently, oxolinic acid and flumequine, synthetic compounds for which plasmid-mediated resistance does not occur, have been used (Michel *et al.*, 1980; Austin, Rayment and Alderman, 1983). These drugs are effective at dosages of 10 mg/kg of fish/day for 10 days and 6 mg/kg of fish/day for 6 days, respectively (*Table 20.3*).

MYCOBACTERIOSIS AND NOCARDIOSIS

Infections by these acid-fast bacteria are notoriously difficult to combat. It is regarded as impractical to treat food fish (Dulin, 1979). However, some drugs have been suggested for the treatment of valuable exotic fish (*Table 20.2*).

SALMONID BLOOD SPOT

According to Llewellyn (1980), the condition in the hatchery is controlled in 10–14 days by administering methylene blue for 5 days in the food at a rate of 1 g of drug/kg of food, followed by 10 days' treatment with oxytetracycline ('3 g of pure terramycin/45 kg of fish/day in the diet'). Finally, methylene blue was administered for a further 5 days.

STREPTOCOCCOSIS

Apart from conventional antibiotic therapy with ampicillin, erythromycin, oxytetracycline and tetracycline, use of a novel fisheries compound, sodium nifurstyrenate at 50 mg/kg of fish/day for 3–5 days has been described (Kashiwagi *et al.*, 1977).

VIBRIOSIS

As a result of a comparison of over 200 compounds, the effectiveness of 5,7-dichloro-8-hydroxyquinoline (25 mg/kg of fish/day for 10 days) has been described for the control of vibriosis (Austin, Johnson and Alderman, 1982). This was found to be more effective than many of the previously recommended drugs (*Table 20.2*).

Conclusion

It may be concluded that sensible use of antimicrobial compounds will control many of the bacterial diseases of fish. The drugs should be prescribed prudently, with clear indications as to the correct means of application. The full dosage must be administered to ensure total inactivation of the pathogen. If a few cells survive, they may serve as a reservoir for resistance transfer in the aquatic milieu.

The reference to proprietary products in this chapter should not be construed as an official endorsement of these products, nor is any criticism implied of similar products which have not been mentioned.

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PRODUCTION OF ANTIBIOTICS BY CUTANEOUS FUNGI AND BACTERIA

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Introduction

Our knowledge of antibiotic production *in vitro* and *in vivo* by members of the skin flora grows slowly but steadily. It is convenient to describe findings under two headings—dermatophytes and bacteria—although in one ecosystem these are slowly being synthesized into a coherent picture of cutaneous ecology. Except in this one narrow field, the ecology of foot infection in man, clinical studies lag behind laboratory aspects. It should be emphasized that interaction can occur between organisms by mechanisms other than antibiosis; such studies are well reviewed in the book by Aly and Shinefield (1982).

Antibiotic production

THE DERMATOPHYTES

Dermatophytes are fungi that invade human and animal skin, causing varying degrees of infection. The range of dermatophytes which are known to produce antibiotics is gradually increasing, as is also our knowledge of the type of antibiotic produced. *Epidermophyton floccosum*, *Microsporum canis*, *M. cookei*, *M. gypseum*, *Trichophyton equinum*, *T. erinacei*, *T. gallinae*, *T. mentagrophytes*, *T. rubrum*, *T. terrestre* and *T. verrucosum* are among those known to produce penicillins, 6-amino-penicillanic acid, fusidic acid and closely related compounds, azolomycin-like antibiotics, actinomycin-like antibiotics, as well as a range of novel compounds with antibacterial, antifungal and anticancer activity (Peck and Hewitt, 1945; Uri, Szathmany and Herpay, 1957; Nishio, 1958; Katagiri and Sato, 1961; Cole, 1966; Wallerström, 1967; 1968; Reith, 1968; Elander *et al.*, 1969; Gip and Palsson, 1970; Youssef *et al.*, 1978; Floersheim *et al.*, 1982).

In the series which includes Youssef's study and subsequent work (W.C. Noble, unpublished) we have found that over 80% of *T. mentagrophytes*, over 50% of *T. rubrum* and over 60% of *E. floccosum* from human sources produce penicillins *in vitro*, while other, non-penicillin antibiotics are produced by more than 50%, 25% and 60% of strains, respectively. It has been rare to find that, *in vitro*, production of non-penicillin antibiotics occurs in the absence of

penicillin production. Nevertheless there is a temperature range preference, penicillin production being optimal at about 26°C, whereas other antibiotics are produced optimally at about 33°C.

Although initial *in vitro* studies have mostly been made in fluid media, often in comparative bulk, attempts have been made to approach *in vivo* conditions more closely. The two most successful approaches have been to use pig skin prepared commercially for burns therapy or to collect human stratum corneum on adhesive tape such as Steridrape (Knight, 1972; Youssef *et al.*, 1978). Under these conditions penicillin and other antibiotics can be shown to be produced on natural substrate, if under unnatural conditions.

The main interest, however, must lie in the *in vivo* production of antibiotic. Experimentally explanted pieces of skin from human or animal infection have been used to demonstrate penicillin production (Uri, Szathmany and Herpay, 1957; Smith and Marples, 1964). Sticky tape samples from human skin (Youssef *et al.*, 1979) showed that penicillin was present on the skins of about one-quarter of patients infected with producer fungi but not receiving any therapeutic antibiotic. Demonstration that penicillin is present depends on showing that the removed stratum corneum is inhibitory to penicillin-sensitive, but not to penicillin-resistant, indicator bacteria and that this inhibitor is destroyed by penicillinase. Since enzymes that destroy other antibiotics are less readily available, it has proved difficult to determine which other antibiotics are formed *in vivo*, although inhibitors resistant to penicillinase can be demonstrated.

There is thus abundant evidence that dermatophytes produce several antibiotics *in vitro* and that at least penicillin is produced *in vivo*.

THE BACTERIA

Production of antibiotics by cutaneous bacteria has also been studied fairly extensively. There are two main classes of substance produced. One is the group of (probably) cyclic peptides with molecular weights of the order of 800–1200 dalton; the other comprises the much higher molecular weight substances, some of which, at least, are bacteriocins.

Hsu and Wiseman (1972) reported that cutaneous staphylococci produced cyclic peptides and we can infer that others have discovered similar substances (Loeb, Moyer and Murray, 1950; Selwyn, Marsh and Sethna, 1976; Holland, Cunliffe and Eady, 1979; Wright and Terry, 1981). *Brevibacterium* species produce similar compounds (Al-Admawy and Noble, 1981). The precise proportion of strains that produce antibiotics is not known, for many workers have been content to report 'inhibitor' production without formally identifying the compounds in more than a few instances. Inhibitor production has been variously reported at 5–10% or higher (Hsu and Wiseman, 1972; Selwyn, Marsh and Sethna, 1976). However, the reservation made by Holland, Cunliffe and Eady (1979) must be borne in mind; this was that inhibition may occur through a number of non-antibiotic compounds such as peroxides or simple organic acids, especially if media with a high sugar content are used. Care is thus needed in interpretation.

In the study reported by Noble and Willie (1980a), 'deferred antagonism' was the criterion of antibiotic production; this demands that a compound

stable for at least 24 h be deposited in an agar growth medium. Some of these strains were later shown to produce a (cyclic) peptide of molecular weight about 1000 dalton. On this basis, only 1.6% of skin strains produced 'antibiotics'. Holland, Cunliffe and Eady (1979) found a comparable proportion of cocci to produce inhibitors active against other cocci, but that the anaerobic coryneform *Propionibacterium acnes* was inhibited by 9.5% of cocci and about 40% of other propionibacteria. At least two mechanisms of antibiosis are extant in cocci.

Production of antibiotic and of bacteriocins *in vivo* has been demonstrated by Selwyn, Marsh and Sethna (1976) and by Noble and Willie (1980b). The latter authors showed that, in a skin surface model of colonization in mice, staphylococci which produce peptides *in vitro* would outgrow indicator strains of *Staphylococcus aureus*; non-producers showed no such ability to outgrow indicators. In a sub-surface injection model in mice, however, the producers (which were mainly 'non-pathogenic' *S. epidermidis* strains) failed to compete with the pathogenic *S. aureus*. It seems probable that this represents the inability of non-pathogens to persist against the body defence mechanisms, rather than that antibiotic was not produced in a subcutaneous site.

Selection of antibiotic resistance

It is logical to suppose that one result of antibiotic production is to reduce the skin flora and also that such bacteria as persist are necessarily resistant to the antibiotic.

Youssef *et al.* (1979) showed that there were significantly fewer bacteria in lesions of humans caused by producer fungi than in those caused by non-producer fungi (Table 21.1). One function of antibiotic production *in vivo* is to reduce competition for substrate or for adhesion sites.

Bibel and LeBrun (1975) found that penicillin-resistant bacteria increased in number during experimental infection with *Trichophyton* spp. in man. Smith and Marples (1964) had earlier described the hedgehog as a source of penicillin-resistant staphylococci during infection with *T. erinacei*. Smith (1965) later denied a causal correlation, however, claiming that uninfected hedgehogs also frequently carried resistant cocci.

In the study by Youssef *et al.* (1979), it was shown that selection of resistant bacteria occurred when a producer fungus was present. In natural lesions of humans there was an excess of resistance to penicillin, fusidic acid and tetracycline, but not to streptomycin, among bacteria isolated from fungal infection (Table 21.2).

Table 21.1 QUANTITATIVE BACTERIAL COUNTS IN LESIONS OF TINEA

	Bacterial count/cm ² , percentage distribution			Total samples
	< 10 ²	10 ² -10 ³	< 10 ³	
Antibiotic-producing fungus:				
present	76	24	0	25
absent	42	37	21	19

Source: after N. Youssef (personal communication.)

Table 21.2 ANTIBIOTIC RESISTANCE IN BACTERIA FROM DERMATOPHYTE LESIONS CAUSED BY PENICILLIN-PRODUCING AND NON-PRODUCING FUNGI

	Percentage with principal bacterial flora resistant to ^a						Total samples
	P	A	T	F	S	M	
Antibiotic-producing fungus:							
present	84	80	68	56	36	16	25
absent	47	47	26	26	16	16	20
χ^2	<0.01	<0.025	<0.01	<0.05	NS	NS	

Source: after Youssef *et al.* (1979).

^aP, benzyl penicillin; A, ampicillin; T, tetracycline; F, fusidic acid; S, streptomycin; M, methicillin.

In vitro, growth of two producer Trichophytons on human stratum corneum and on pig skin, resulted in the selection of *S. aureus* strains bearing a penicillinase plasmid from mixtures of staphylococci isogenic but for that plasmid (Ryall, Holt and Noble, 1981). These authors found that penicillin production was most vigorous at the advancing edge of fungal colonies on agar and that selection of resistant cocci was most pronounced at the edge. It should be noted, however, that at the centre of the colony, not only was less penicillin produced by the fungus, but the production of penicillinase by the resistant staphylococci was sufficient to permit the growth of sensitive cells. W.C. Noble (unpublished) has extended these observations in a mouse model of infection in which, in dermatophytes lesions, the percentage of resistant cocci rises in 48h from about 10% to about 100% if a penicillin producer fungus is present, but remains approximately constant in a lesion caused by a non-producer.

Selection of resistance to bacterial peptide antibiotics has not been studied using producer cocci, but Bibel, Smiljanic and Lovell (1978) reported that, in human, artificially colonized by *Bacillus licheniformis* strains which produce bacitracin (a similar antibiotic to those produced by the cocci), bacitracin resistance increased among members of the normal skin flora. It is worth recording that probiotic action was also seen.

Ecological and clinical changes

Interaction as a therapeutic tool has been used most intensively in man with the 502A strain of *S. aureus* (Aly, Shinefield and Maibach, 1982) and by Wickman (1982), but strain 502A is not an antibiotic producer. Attempts to suppress infection in humans using antibiotic-producing non-pathogens has generally been unsuccessful (Noble, 1982), presumably due to the inability of 'non-pathogens' to persist subcutaneously. The most successful studies are those on *Dermatophilus congolensis* reported by D.H. Lloyd (Chapter 22).

In human foot infection there is, however, a model in which interaction by antibiotic production apparently takes part (Leyden, 1982). The simplest form of foot infection, in which dermatophytes are almost always recoverable, is the dry, scaly, non-malodorous athlete's foot. If the foot is fairly dry, the infection does not progress but, if the foot becomes wet, Gram-positive bacteria, especially *Brevibacterium* spp., may take over producing a soggy, macerated, malodorous lesion. It is presumed that antibiotic production by

the fungi results in selection of the penicillin-resistant *Brevibacteria*. These are highly proteolytic, which accounts for the skin damage, and produce CH_3SH from methionine, which accounts for the malodour; this gas is also toxic to fungi and this may account for the difficulty experienced in recovering dermatophytes from these lesions. There is thus a spectrum of disease in tinea pedis.

Implications of antibiotic production

Several aspects of antibiotic production by cutaneous microbes command attention. The skin of man and animals is a habitat in which some common antibiotics are produced naturally, resulting in the selection of a resistant bacterial flora. It seems probable that, in evolutionary terms, antibiotic production was followed by the evolution of antibiotic resistance, contributing to the pool of resistance genes and mechanisms for their transfer (Chapter 23). It should be possible to use natural antibiosis as a therapeutic tool or to use the knowledge of interaction as a weapon in the prevention of lesion progression.

More detailed clinical studies in man and in both experimental and commercial animals are required.

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INTERACTION BETWEEN ANTIBIOTIC-PRODUCING BACTERIA AND *DERMATOPHILUS CONGOLENSIS*: A POTENTIAL THERAPEUTIC TOOL?

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Introduction

The treatment of bacterial skin disease in farm animals raises a variety of problems. Topical therapy is limited by the thickness of the coat and the ability of the infected animal or its herd mates to lick off the medicament. Where the infection is deep seated or provokes epidermal thickening and scab formation, topically applied drugs are unlikely to penetrate effectively into the affected area. Their use is labour intensive and may therefore be uneconomic. Antibiotics such as penicillin and streptomycin are also unsuitable for topical use because of their ability to induce hypersensitivity reactions.

Parenteral therapy also presents difficulties. Although drugs present in the blood are readily transported into the dermal tissue, this is not true of the avascular epidermis. High dose rates and/or prolonged treatment are thus required for the effective treatment of epidermal infections. The range of drugs which may be used in high doses is limited by toxicity and cost. They may have adverse effects on the normal flora both of the skin and other organs, and the suppression of the normal skin flora may in turn permit colonization by resistant organisms or the selection of resistant strains from within the resident populations (Marples and Williamson, 1969; Marples and Kligman, 1971). The ability of such resistant commensal organisms subsequently to transmit resistance factors to pathogens (see Chapter 23) represents an additional hazard. Antibacterial drug therapy suffers from the additional disadvantage that, if no immunity is developed to the disease, it may recur when drug administration ceases. This is a particular problem in *Dermatophilus congolensis* infection (Blancou, 1976). There is thus a need for cheaper and more effective methods for the treatment and control of bacterial skin diseases in farm animals.

A biological approach to the control of skin disease

The primary barrier to skin infection is formed by the superficial layers of the stratum corneum and the constituents of the sweat and sebaceous secretions which permeate them (Jenkinson, 1980). The normal skin microflora which

is found in this region also contributes towards skin defence by occupying the available ecological niches and inhibiting colonization by 'foreign' bacteria (Lloyd, 1980). The nature of this antagonistic activity is as yet poorly understood, but is probably dependent in part on the release of inhibitory chemicals. Both coagulase-positive and coagulase-negative staphylococci are known to produce antibiotics (Halbert, Swick and Sonn, 1953; Barrow, 1963; Selwyn, 1975) which are effective chiefly against other Gram-positive bacteria. Inhibitory activity may also be mediated by the production of bacteriocins (Reeves, 1965) which have a narrower spectrum of activity and are normally active only against members of the same or closely related species. It is evident that the normal Gram-positive skin flora is also responsible for inhibiting colonization by other types of bacteria. Treatment of skin with substances which depress the normal microflora leads to colonization by Gram-negative bacteria (Shehadeh and Kligman, 1963).

In human medicine, bacterial antagonism has been employed against outbreaks of skin disease caused by some virulent strains of *Staphylococcus aureus*. Patients deliberately colonized with a low virulence strain, 502A, were protected from the disease and only trivial and short-lived lesions occurred (Light *et al.*, 1967; Boris, 1968). Unfortunately, 502A did not give protection against distantly related strains of *S. aureus* and it also caused abscess formation and septicaemia in some patients (Drutz *et al.*, 1966; Houck, Nelson and Kay, 1972). Antibiotic-producing, coagulase-negative staphylococci are known to have an inhibitory effect on skin infection with *S. aureus* in laboratory animals (Selwyn, Marsh and Sethna, 1976; Noble and Willie, 1980) and have the advantage of being generally non-pathogenic and therefore safer for clinical use. However, their effectiveness under clinical conditions has yet to be demonstrated.

Bacterial antagonism and *Dermatophilus congolensis* infection

Dermatophilus congolensis AS A SKIN PATHOGEN

Studies of bacterial antagonism on the skin surface with both coagulase-positive and coagulase-negative staphylococci have concentrated on their interaction with *S. aureus* (Noble, 1981). However, experimental induction of lesions with *S. aureus* cannot readily be achieved on the intact skin. Such experimental studies have therefore generally relied on changes in the ratio of pathogen to inhibitor of mixed inocula placed on the skin and have not measured the ability to prevent staphylococcal disease.

Characteristic skin lesions can, however, readily be induced by infection with the actinomycete *D. congolensis* (Lloyd and Jenkinson, 1980; Lloyd and Noble, 1982), a specific skin pathogen which causes economically important diseases in a variety of farm animals (Lloyd and Sellars, 1976). Under natural conditions infection with this organism occurs in wet weather when the motile infective stage, the 'zoospore', is attracted to areas of weakness in the superficial stratum corneum and germinates, giving rise to a filament which penetrates and grows within the living epidermis (Roberts, 1967). Infiltration of neutrophils and hyperkeratosis in the affected region results in the formation of typical exudative lesions with scab formation which is apparent at 48–96 h post-infection. Although normal skin is relatively resistant to *D. congolensis*

infection, susceptibility is greatly increased by prolonged or intense rainfall (Austwick, 1976) and these effects can be simulated in experimental infections by treatment of the skin with organic solvents (Lloyd and Jenkinson, 1980).

Dermatophilus congolensis is generally susceptible to a wide range of conventional antibiotics (Abu-Samra, Imbabi and Mahgoub, 1976), but treatment of the diseases which it causes (Blancou, 1976) is complicated by its location within the avascular epidermis, beneath a well-developed scab. In addition, no immunity develops following infection (Roberts, 1967) and thus successfully treated animals are susceptible to reinfection. *Dermatophilus congolensis* is, however, susceptible to inhibitory substances produced by staphylococci (S.N. Appiah, unpublished observations) and must pass through the superficial epidermis in which the normal skin flora resides (Montes and Wilborn, 1970; Lloyd, Dick and Jenkinson, 1979) in order to reach its habitat in the living epidermis. It is therefore a potential candidate for control by means of antibiotic-producing skin commensals which may be able to inactivate the infective zoospores as they pass through the stratum corneum.

INHIBITION OF *Dermatophilus congolensis* BY ANTIBIOTIC-PRODUCING STAPHYLOCOCCI

The ability of antibiotic-producing staphylococci to inhibit infection with *D. congolensis* has been tested *in vivo* in the mouse model developed by Lloyd and Noble (1982). Inoculation was carried out on the clipped and ether-washed skin of the lumbosacral region of haired mice. Suspensions of highly motile *D. congolensis* zoospores (10^{12} – 10^{13} viable units/ml) were applied to the skin following treatment with either cetomacrogol cream alone or cream containing antibiotic-producing or non-producing control staphylococci

Table 22.1 BACTERIAL STRAINS

Action	Species	Identification	
Pathogen	<i>Dermatophilus congolensis</i>	W3197	Received from Dr W. Kaplan, C.D.C., Atlanta, Georgia
Antibiotic producers	<i>Staphylococcus epidermidis</i>	S6+	(Selwyn, Marsh and Sethna, 1976)
	<i>S. capitis</i>	3931	(Noble and Willie, 1980)
	<i>S. aureus</i>	H5	
Controls	<i>S. aureus</i>	Wood 46	(NCTC 7121)
	<i>S. aureus</i>	PS42E	(NCTC 8357)
	<i>S. simulans</i>	7944	(NCTC 7944)

(Table 22.1) at a density of 10^8 – 10^{12} cells/g. Penetration of the stratum corneum by *D. congolensis* resulted in the formation of scabs which were examined after 48 h and scored as the percentage of inoculated area occupied by lesions.

Control mice inoculated with cream only or the suspensions of staphylococci did not develop such lesions (Noble, Lloyd and Appiah, 1980). Inhibition of lesion formation by *D. congolensis* was obtained with two of the three

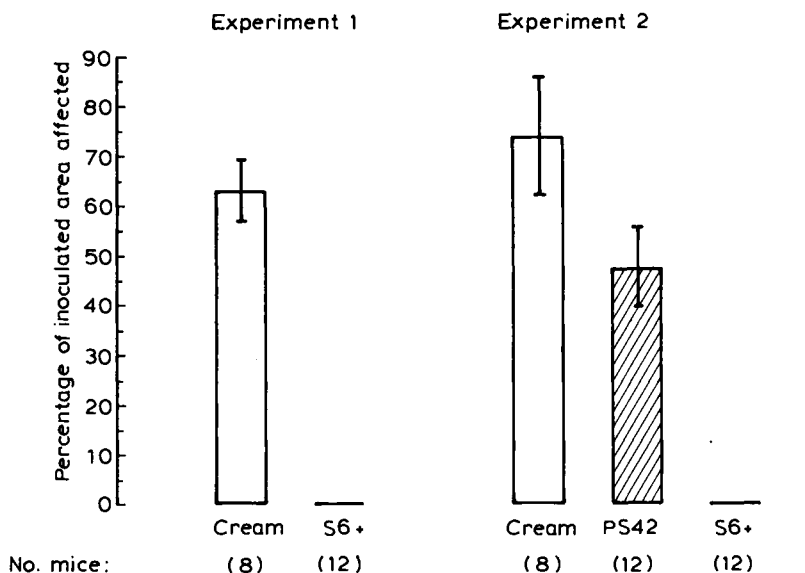


Figure 22.1 Mean percentage area ($\pm$ s.e.) of inoculated mouse skin developing characteristic lesions following various treatment regimens in two of four experiments (see also Figure 22.2). All sites were inoculated with *Dermatophilus congolensis* after application of cream only ($\square$, cream) or cream incorporating different bacteria (see Table 22.1; $\square$, antibiotic non-producers) (from Noble, Lloyd and Appiah, 1980)

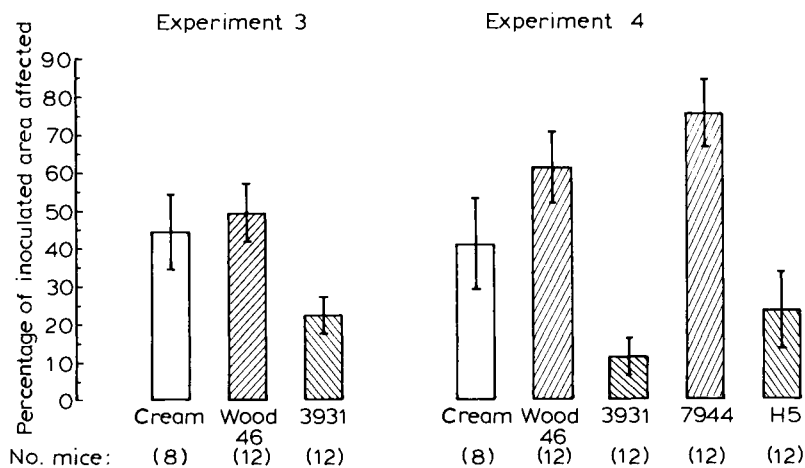


Figure 22.2 Mean percentage area ($\pm$ s.e.) of inoculated mouse skin developing characteristic lesions following various treatment regimens in two of four experiments (see also Figure 22.1). All sites were inoculated with *Dermatophilus congolensis* after application of cream only ($\square$, cream) or cream incorporating different bacteria (see Table 22.1; $\square$, antibiotic producers; $\square$, non-producers) (from Noble, Lloyd and Appiah, 1980)

antibiotic producers. The effect with the *S. epidermidis* strain S6+ was almost complete (Figure 22.1), but *S. capitis* 3931, which is known to be a less potent antagonist *in vitro* (W.C. Noble, unpublished studies) was less effective (Figure 22.2). Use of the *S. aureus* strain H5 was associated with a lower mean level of lesion formation, but this effect was not significant. Strain H5 is known to produce a high molecular weight bacteriocin which is possibly less effective against *D. congolensis* than the polypeptide antibiotics produced by S6+ and 3931 (Noble and Willie, 1980). Interesting effects were also obtained with the non-antibiotic-producing control organisms. *Staphylococcus aureus* PS42E was shown to reduce the activity of *D. congolensis* (Figure 22.1), whereas Wood 46 had no effect (Figure 22.2). In contrast, *S. simulans* 7944 was associated with a considerably enhanced level of lesion formation (Figure 22.2). The nature of the interactions between the bacteria on the skin surface which accounts for these effects is not understood, but the increased pathogenicity associated with the presence of 7944 may involve chemotropic attraction of the zoospores of *D. congolensis* towards CO₂ (Roberts, 1967) produced by the staphylococci, or perhaps the production of a probiotic (Selwyn and Ellis, 1972).

POTENTIAL USE OF ANTIBIOTIC PRODUCERS AGAINST *Dermatophilus congolensis* INFECTION

The effects observed in this study were obtained when *D. congolensis* was inoculated only a short time after the inhibitor organisms. Quantitative studies have shown that high populations of these inhibitors persist on mouse skin for at least 4 days following application in cream (D.H. Lloyd and W.C. Noble, unpublished observations) and it seems likely that they could afford protection for longer periods than those demonstrated in this study. The organisms tested were all human isolates and it is possible that longer term colonization could be obtained with strains indigenous to the animal species inoculated. The existence of bacteria inhibitory to *D. congolensis* in cattle has already been reported (Fraser and Horne, 1967, cited by Lloyd, 1978). Evidence of the ability of inhibitor-producing *Pseudomonas* spp. to colonize sheep skin and suppress other bacteria is available from studies of fleece rot (Merritt and Watts, 1978; Merritt, 1980).

The prospect of biological control of *D. congolensis* infection by means of bacterial interference is very attractive. The method is, in theory, cheap and self-renewing. However, its potential remains to be tested in longer term studies and in the field.

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TRANSMISSION OF GENES BETWEEN STAPHYLOCOCCI ON SKIN

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Introduction

In the past, the transfer of antibiotic resistance between staphylococci was thought to occur by transduction, a process whereby phage propagated on the donor strain was mixed at high concentration with cells of the recipient *in vitro*. However, there was argument on the likelihood of this process occurring *in vivo*, although some workers (Jarolmen, Bondi and Crowell, 1965; Novick and Morse, 1967) achieved resistance transfer experimentally in mice using this method. The discovery that antibiotic resistance plasmids could be transferred in mixed cultures (Lacey, 1971a; 1971b; 1980a; 1980b; Naidoo and Noble, 1978; Meijers, Winkler and Stobberingh, 1981) provided a better explanation for *in vivo* events. Transfer in mixed culture is thought to be phage-mediated (Lacey, 1971a, 1971b; Meijers, Winkler and Stobberingh, 1981), but cell-to-cell contact is required for transfer and although free phage particles have not been detected in the cultures, cell-bound phage particles have been implicated in a conjugative-type process (Lacey, 1980a).

Recently, a new conjugative process has been described in which phage appears to play no role (Forbes and Schaberg, 1983; McDonnell, Sweeney and Cohen, 1983). Resistance plasmids were transferred from donor to recipient in a conjugation process similar to that in Gram-negative bacteria. Our own work confirms these observations and previous reports on the transfer of gentamicin resistance between staphylococci on skin (Naidoo and Noble, 1978; 1981; Jaffe *et al.*, 1980) may be attributed to this transfer mechanism. In the latter two reports, antibiotic resistance plasmids were transferred from human coagulase-negative staphylococci to *Staphylococcus aureus*. The normal skin flora which is largely composed of different species of coagulase-negative staphylococci and coryneforms may thus act as a reservoir of resistance plasmids capable of being transmitted to pathogenic *S. aureus* (Lacey, 1975). This raises the question whether resistance genes can be transferred from members of the normal human skin flora to strains potentially pathogenic to animals. For example, man and his dog are often in close contact and the human and canine skin microflora may be transiently exchanged, providing a situation where transfer events could occur between the different species of staphylococci. Attempts were therefore made to see if antibiotic

resistance could be transferred from human coagulase-negative staphylococci to canine *S. aureus* *in vitro* and *in vivo* on skin.

Transfer of antibiotic resistance from human coagulase-negative staphylococci to human *Staphylococcus aureus*

Thirty-five multiply antibiotic-resistant clinical isolates of coagulase-negative strains, originally selected because they were resistant to gentamicin, were incubated in mixed broth culture with three *S. aureus* recipients. Characteristics of the *S. aureus* recipient strains are given in *Table 23.1*. Twelve of the 35 strains were able to transfer one or more antibiotic resistances to at least two of the three recipients used. Of the 12 coagulase-negative donor strains, six were strains of *S. hominis*, four were *S. epidermidis* and two were *S. haemolyticus*. Details of two of the strains which transferred antibiotic resistance at highest frequency and which were used in further experiments are

Table 23.1 CHARACTERISTICS OF *Staphylococcus aureus* RECIPIENTS USED AND COAGULASE-NEGATIVE DONOR STRAINS OF HUMAN ORIGIN

Strain	Source	Phage-type	Antibiotic resistance ^a
<i>S. aureus</i> recipients			
B111	Impetigo lesion (non-hospital)	3A/53/85	Pc
8325	NCTC known plasmidless strain	47/53/75/77/84/85	—
80CR5	Engel <i>et al.</i> (1980) Restriction deficient mutant	Groups I, II, III, IV	Pc
<i>Coagulase-negative staphylococci</i>			
<i>S. epidermidis</i> 100724	C.S.F (USA)	NT	Pc, Tc, Nm, Em, Gm, Chl
<i>S. hominis</i> BW	Skin (St. John's Hospital, London)	NT	Pc, Tc, Nm, Em, Gm

^aPc, resistance to penicillin; Tc, resistance to tetracycline; Nm, resistance to neomycin; Em, resistance to erythromycin; Gm, resistance to gentamicin; Chl, resistance to chloramphenicol. NT, non-typable with standard set of *S. aureus* typing phages.

given in *Table 23.1*. In all 12 coagulase-negative donor strains, gentamicin resistance was always transferred preferentially.

When the *S. hominis* BW and *S. epidermidis* 100724 strains were mixed with the *S. aureus* recipients and occluded on skin (Naidoo and Noble, 1978), resistance transfer occurred at higher frequency than in the same cultures incubated in broth. *Table 23.2* shows transfer of gentamicin, tetracycline and erythromycin resistances to *S. aureus* 80CR5. The antibiotic resistances were transferred at a 100-fold greater frequency on skin. Only the erythromycin resistance determinant of the *S. hominis* BW was not transferred. This resistance was not plasmid-mediated. Skin appeared to facilitate the transfer of resistance plasmids from coagulase-negative staphylococci to *S. aureus*.

The mechanism of transfer is probably a conjugative-type process. However, unlike previous reports on transfer in mixed culture (Lacey 1971a; 1971b; Lacey, 1980a; 1980b; Meijers, Winkler and Stobberingh, 1981), phage does not appear to be involved. Broth filtrates do not transfer resistance and

Table 23.2 TRANSFER OF ANTIBIOTIC RESISTANCE FROM COAGULASE-NEGATIVE STAPHYLOCOCCI TO *Staphylococcus aureus*

Donor	Recipient	Site of transfer	Time	Frequency of transfer ^{a,b}		
				Gm	Tc	Em
<i>S. hominis</i> BW	<i>S. aureus</i> 80CR5	Broth	6h	1×10^{-8}	2×10^{-9}	5×10^{-10}
		Mouse ^c	6h	7×10^{-6}	5×10^{-7}	0
<i>S. epidermidis</i> 100724	<i>S. aureus</i> 80CR5	Broth	6h	8×10^{-8}	2×10^{-9}	3×10^{-9}
		Mouse ^c	6h	4×10^{-6}	3×10^{-7}	7×10^{-7}
		Human forearm	6h	4×10^{-6}	3×10^{-7}	4×10^{-7}

^a Number of transconjugants per final number of recipients.^b Symbols as in Table 23.1.^c Mean of 2 mice.

transfer is not abolished by sodium citrate (Table 23.3). DNase has no effect, ruling out transformation, but incubation of the cultures on filters enhances the frequency of plasmid transfer. Cell-to-cell contact is therefore required for the transfer of resistance plasmids between the staphylococci. Skin, the natural habitat of these organisms, facilitates the transfer effect thus providing the most suitable environment for the transmission and spread of antibiotic resistance between strains of non-pathogenic and pathogenic staphylococci.

Table 23.3 TRANSFER OF GENTAMICIN RESISTANCE PLASMIDS FROM *Staphylococcus hominis* BW AND *S. epidermidis* 100724 TO *S. aureus* B111

Mixed culture incubation (24h)	Frequency of transfer ^a	
	Donor BW	Donor 100724
Control	4×10^{-7}	1×10^{-7}
-Ca ²⁺ ^b	7×10^{-8}	3×10^{-8}
+DNase (100 mg/l)	1×10^{-7}	2×10^{-7}
On filters	6×10^{-5}	2×10^{-5}
Broth filtrate + recipient	0	0

^a Number of transconjugants per final number of recipients.^b Calcium ions removed by addition of 0.03mol sodium citrate.

Transfer of antibiotic resistance from human coagulase-negative staphylococci to canine *Staphylococcus aureus*

The gentamicin resistance plasmids in the coagulase-negative strains play an important role in the conjugative process (data unpublished). Preliminary experiments have shown that these elements are responsible for mobilizing the tetracycline and erythromycin resistance plasmids in the host. Absence of such conjugative elements in strains used in transfer experiments may have been responsible for the failure in the past to demonstrate transmission of resistance between staphylococci of human and animal origin (Lacey, 1980b). It would seem more appropriate for a conjugative transfer system to operate between human and animal staphylococci rather than phage-mediated transduction where very non-specific phage would be necessary.

To see if plasmid transfer could occur between the human *S. hominis* BW

Table 23.4 HUMAN COAGULASE-NEGATIVE STRAINS CAPABLE OF TRANSFERRING GENTAMICIN RESISTANCE TO CANINE STAPHYLOCOCCI

Strain	Species	Source	Antibiotic resistance ^a
J580	<i>Staphylococcus haemolyticus</i>	Skin (St. John's Hospital, London)	Tc, Nm, Gm
J225	<i>S. hominis</i>	Skin (St. John's Hospital, London)	Pc, Nm, Gm
J542	<i>S. hominis</i>	Skin (St. John's Hospital, London)	Tc, Em, Nm, Gm
3/02477(1)	<i>S. epidermidis</i>	Urine (Hope Hospital, Manchester)	Pc, Tc, Em, Nm, Gm
3/02477(2)	<i>S. haemolyticus</i>	Urine (Hope Hospital, Manchester)	Pc, Tc, Nn, Gm

^a Symbols as in Table 23.1.

or *S. epidermidis* 100724 strains and canine *S. aureus*, the donor strains were incubated in mixed culture with 43 epidemiologically distinct isolates of canine *S. aureus*. Twenty-one of the strains acquired the gentamicin resistance plasmid from the *S. epidermidis* and 14 from the *S. hominis*. Thus, canine staphylococci do have the potential to conjugate with human staphylococci and acquire resistance. Three of the canine *S. aureus* recipients could also acquire gentamicin resistance from five other human coagulase-negative strains tested (Table 23.4) at frequencies of about 10^{-6} . Transfer of gentamicin resistance from one of these donors—*S. haemolyticus* J580—to one of the canine recipients was attempted *in vivo* on dog skin. The hair on the abdominal region was clipped and the mixed cultures applied to the skin with a cotton-tipped swab. The area was then occluded for 5 h using Steridrape. On adjacent areas, the donor and recipient cultures were applied individually as controls. After 5 h occlusion, the skin was sampled by the scrubbing technique of Williamson and Kligman (1965). A similar experiment was carried out with human *S. epidermidis* 100724 to see if tetracycline resistance could be transferred on dog skin. Results for both experiments are given in Table 23.5. Transfer of both gentamicin resistance and tetracycline resistance occurred at higher frequency on dog skin compared to the same cultures in broth.

Table 23.5 *In vivo* TRANSFER OF ANTIBIOTIC RESISTANCE FROM HUMAN STAPHYLOCOCCI TO CANINE *Staphylococcus aureus*

Donor (human)	Recipient (canine)	Site of transfer	Time	Resistance transferred ^a	Frequency of transfer ^b
<i>S. haemolyticus</i> J580	20/279R	Dog	5h	Gm	5×10^{-6}
<i>S. haemolyticus</i> J580	20/279R	Broth	5h	Gm	3×10^{-7}
<i>S. epidermidis</i> 100724	25/579R	Dog	5h	Tc	1×10^{-4}
<i>S. epidermidis</i> 100724	25/579R	Broth	5h	Tc	3×10^{-8}

^a Symbols as in Table 23.1.^b Number of transconjugants per final number of recipients. No antibiotics applied to skin. No transconjugants obtained when strains applied individually.

To further examine the simultaneous transfer *in vivo* of different resistance markers from the human *S. epidermidis* 100724 to canine *S. aureus*, the cultures were applied to mouse skin, a more convenient *in vivo* model. Unlike transfer to human *S. aureus*, transfer of gentamicin resistance to canine *S. aureus* occurred at lower frequency on mouse skin compared to tetracycline and erythromycin resistances (Table 23.6). In mice 2 and 3, no gentamicin-

Table 23.6 TRANSFER OF ANTIBIOTIC RESISTANCES FROM HUMAN STAPHYLOCOCCI TO CANINE *Staphylococcus aureus*

Donor (human)	Recipient (canine)	Site of transfer	Time	Frequency of transfer ^{a,b}		
				Gm	Tc	Em
<i>S. epidermidis</i> 100724	25/579R	Mouse 1	6h	8×10^{-6}	1×10^{-6}	2×10^{-7}
<i>S. epidermidis</i> 100724	25/579R	Mouse 2	6h	0	5×10^{-6}	2×10^{-7}
<i>S. epidermidis</i> 100724	25/579R	Mouse 3	6h	0	1×10^{-6}	9×10^{-8}
<i>S. epidermidis</i> 100724	25/579R	Broth	6h	1×10^{-9}	2×10^{-9}	3×10^{-10}

^aNumber of transconjugants per final number of recipients.

^bSymbols as in Table 23.1.

resistant transconjugants were recovered, but this may have been due to the lower recovery of cells from skin compared to broth culture. However, both tetracycline and erythromycin resistances were transferred at higher frequency on skin compared to broth cultures. Skin of different animals is therefore able to promote transfer of resistance between staphylococci irrespective of origin, although differences in transfer frequency demand further study.

When individual transconjugants were examined for the acquisition of resistance markers other than the one selected, it was apparent that, as in transfer between staphylococci of human origin, more than one resistance

Table 23.7 CO-TRANSFER OF RESISTANCE PLASMIDS

Resistance selected for (no. tested) ^a	Percentage resistant to:				
	Gm	Tc	Em	Chl	Pc
Em (112)	100	4	41	50	100
Tc (136)	2	100	2	59	10
Em (218)	38	3	100	29	26

^aSymbols as in Table 23.1.

marker could be transferred simultaneously. Table 23.7 shows the co-transfer of gentamicin, tetracycline and erythromycin resistances and, although not selected for, chloramphenicol and penicillin resistance also appeared to have been transferred from the multi-resistant donor. Agarose gel electrophoresis of DNA from the transconjugants showed that the antibiotic resistances were all borne on separate plasmids. Although transferred in combination, the plasmids existed as individual elements in the canine host. Again, this would argue for a conjugation transfer mechanism rather than phage-mediated transduction, as the phage would be required to carry as many as five different plasmids in some cases, simultaneously. Once the resistance plasmids were established in the canine strain they could be further transferred to other canine *S. aureus* and also back to human *S. aureus*.

Transfer of antibiotic resistance from human coagulase-negative staphylococci to *Staphylococcus aureus* of other animal origin

Conjugation and hence transmission of antibiotic resistance plasmids can occur between human and canine staphylococci. The multi-resistant *S. epidermidis* 100724 was also found to be able to conjugate with and transfer resistance plasmids to *S. aureus* from other animal species (Table 23.8). The

Table 23.8 TRANSFER OF ANTIBIOTIC RESISTANCE FROM HUMAN *Staphylococcus epidermidis* 100724 TO *S. aureus* ISOLATED FROM ANIMALS

Donor (human)	Recipient (animal)	Site	Frequency of transfer ^{a,b}			
			Gm	Tc	Em	Chl
<i>S. epidermidis</i> 100724	CRF 89, pig	Filter	2×10^{-7}	5×10^{-8}	6×10^{-8}	$> 10^{-5}$
<i>S. epidermidis</i> 100724	CRF 90, pig	Filter	3×10^{-7}	$> 10^{-5}$	2×10^{-7}	$> 10^{-5}$
<i>S. epidermidis</i> 100724	CRF 285, cow	Filter	3×10^{-7}	2×10^{-7}	1×10^{-7}	$> 10^{-5}$
<i>S. epidermidis</i> 100724	CRF 400, cow	Filter	4×10^{-8}	6×10^{-8}	1×10^{-8}	$> 10^{-5}$
<i>S. epidermidis</i> 100724	CRF 345, hen	Filter	4×10^{-7}	$> 10^{-5}$	2×10^{-7}	$> 10^{-5}$
<i>S. epidermidis</i> 100724	CRF 411, wildfowl	Filter	1×10^{-8}	1×10^{-8}	1×10^{-8}	$> 10^{-5}$

^aNumber of transconjugants per final number of recipients.

^bSymbols as in Table 23.1.

transfer was carried out by incubating the mixed cultures on filters. This was previously shown to enhance transfer compared to broth culture incubation (Table 23.3). Plasmids determining gentamicin, tetracycline, erythromycin and chloramphenicol resistance could all be transferred to *S. aureus* of porcine, bovine and avian origin, indicating that these animal strains also have the potential to accept resistance genes from human strains.

Discussion

These experiments have shown that canine and other animal staphylococcal strains have the potential to acquire antibiotic resistance from human staphylococci. Transfer of resistance between the staphylococci occurs experimentally on skin, the *in vivo* site at which these events probably take place naturally. Whether the resistances will be maintained in the animal host will depend on (1) the frequency at which the transfer events take place, (2) the amount of antibiotic pressure available to select resistance and (3) the stability of the resistance in the new host. Human resistance plasmids transferred into canine strains are very labile and are rapidly lost, leading to sensitive cultures unless grown in the presence of antibiotics which presumably select stable clones. This may explain why so few canine staphylococci are currently multi-resistant. Those that are resistant are most often isolates from ear infections where topical antibiotic preparations are used. Thus, although resistance transfer may occasionally occur between human and animal

strains, persistence of resistant populations will depend on the antibiotic selective pressure present. The Gram-negative bacteria already present a problem with multiple antibiotic resistance. Similar drug resistance plasmids in Gram-negative bacilli in man and domestic pets have been reported (Davies and Stewart, 1978), suggesting either plasmid transfer between the Gram-negative bacteria of man and his pets or a common plasmid pool. The widespread use of antibiotics, together with the intimate contact which is common between owner and pet, may be an important factor in plasmid spread, not only between the enteric bacteria of the two populations, but also the skin flora.

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LISTERIA MONOCYTOGENES: HORMONE INHIBITION

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Introduction

For some four decades the effects of natural and synthetic steroids have been of diverse interest and their effects on microorganisms have been a part of this interest. For example, stilboestrol and its analogues were found to inhibit Gram-positive bacteria by Brownlee *et al.* (1943). Rowson, Lamming and Fry (1953) had also referred to the influence of ovarian hormones on uterine infection. Yotis and his colleagues developed an extensive programme, which included the effects of progesterone (1966), androgens (1968), Enovid (Searle) (1969), Norinyl (Syntex) (1970) and gonadal hormones (1971) on staphylococci, and which was reviewed by Yotis (1972).

We have been involved in a parallel approach using *Listeria monocytogenes*—an organism associated with the death of young animals or the compromised mothers (particularly babies and lambs). Harrison, Seaman and Woodbine (1975) found that oestradiol-17 β inhibited the organism and, even earlier, its haemolysin production. The effects of progesterone on *L. monocytogenes* and its haemolysin was further developed (Nicholson, 1980; Brookes *et al.*, 1981) with synthetic hormone derivatives—ethinyloestradiol and norethindrone acetate—which were found to nullify each other.

Following this lead the effects of oestradiol-17 β with progesterone have been examined (Yates, 1982; Tullett, 1983, but using strain 4379).

Methods

In principle, the methods used were similar to those in the earlier work. Some modifications were made in the light of the experience gained. For example, the hormone concentrations were extended to 40 $\mu\text{g/ml}$ and the time lapse to 48 h; 5 strains were included (NCTC 10357, C52, 4885, 4317 and 5214 as 5214m via Professor H.P.R. Seeliger, Würzburg). Assays of the progesterone were also carried out, 4 hourly, to check on possible inactivation, and minimum inhibitory concentrations (MICs) were also done for the different strains.

Results

The MICs broadly correlated with their haemolytic potential the strains C52, 4379 (highly haemolytic) at 60 µg/ml and 4885, 10357 (lowly haemolytic) at 40 µg/ml, with 5214m in between for oestradiol-17β. Progesterone was similar, but at higher concentrations (50–80 µg/ml).

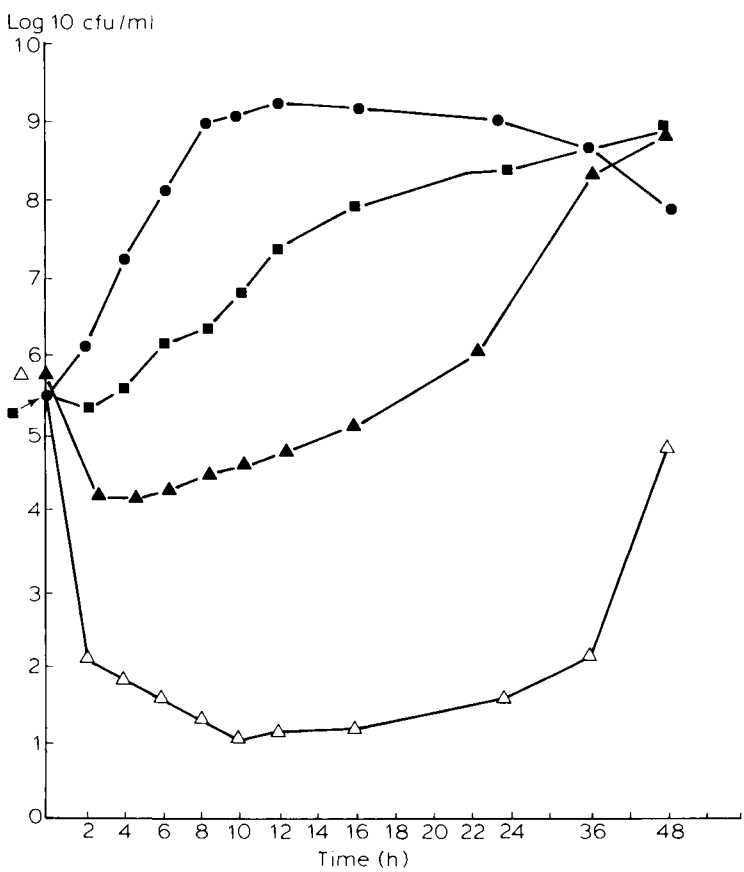


Figure 24.1 Effect of progesterone on growth of *Listeria monocytogenes*: ■, 10 µg/ml progesterone; ▲, 20 µg/ml progesterone; △, 40 µg/ml progesterone; ●, control 0 µg/ml hormone

Figures 24.1–24.5 show the effects on the number of colony-forming units (cfus) of the medium haemolytic 5214m and demonstrate that these occur at lower concentrations: Figure 24.1 depicts the effect of progesterone on growth; Figure 24.2 the effect of oestradiol on growth; Figure 24.3 the effect of combinations on growth; Figure 24.4 the effect on haemolysin production with either hormone; and Figure 24.5 the effect on haemolysin production by combinations.

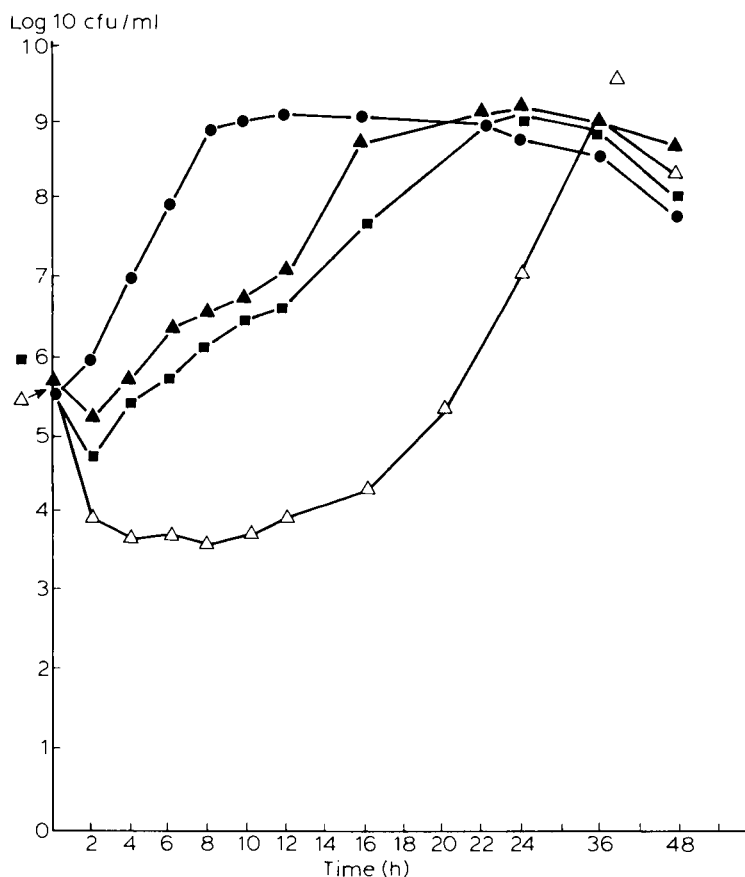


Figure 24.2 Effect of oestradiol on growth of *Listeria monocytogenes*: ■, 10 μg/ml oestradiol-17β; ▲, 20 μg/ml oestradiol-17β; △, 40 μg/ml oestradiol-17β; ●, control 0 μg/ml hormone

Discussion

Instead of the somewhat hoped-for synergic potential being exhibited by combined oestradiol-17β and progesterone, there is antagonism. Either alone inhibits both cells and resulting haemolysin, with increasing concentrations. With the combinations, more haemolysis occurred than with single concentrations.

During the course of this work, some preliminary studies by Professor E. Baulieu and his colleagues on a Roussel-Uclaf product (486) were referred to in a note in *Chemical Engineering News* (17 May 1982, p. 26). Correspondence with Professor Baulieu at the Department of Chemical Biology, Université de Paris Sud, led to his opinion (personal communication, 6 September 1982) that the individual inhibitory effect may be related to membrane action and that a mixture of oestradiol-17β and progesterone might well be expected to offset one another, as this had been observed in the oocyte system.

The present studies are in accord with this opinion. However, they do not

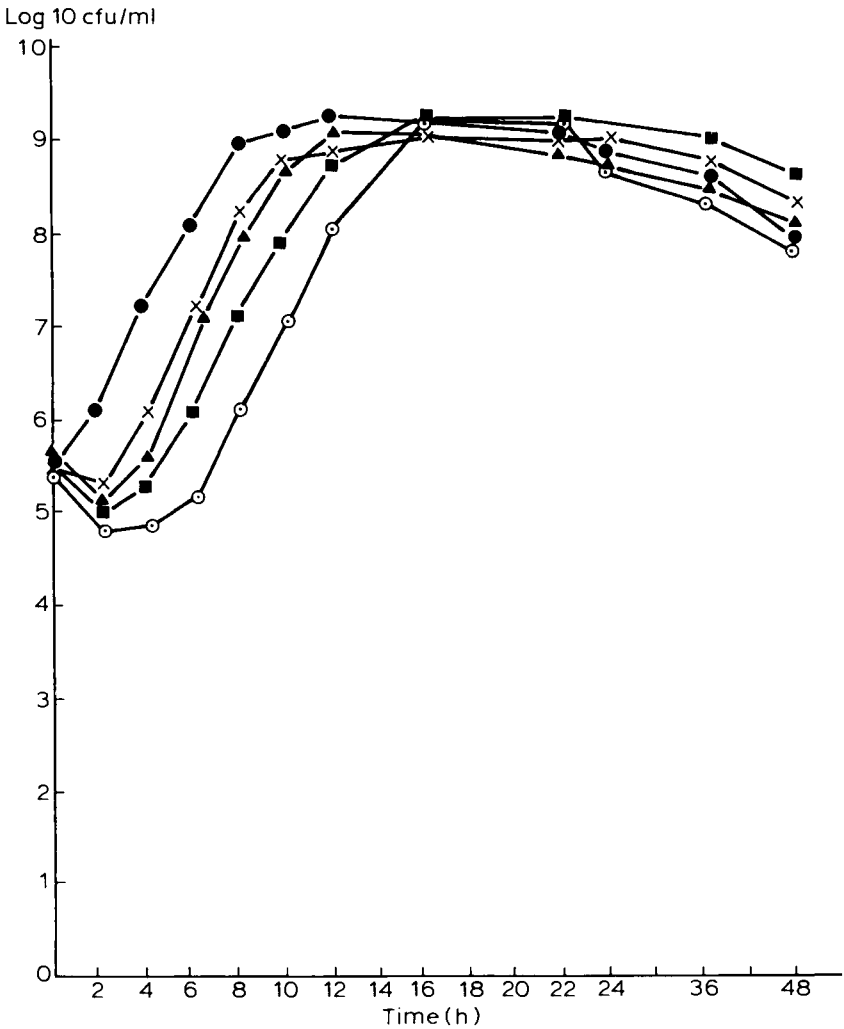


Figure 24.3 Effect of combinations of progesterone and oestradiol on growth of *Listeria monocytogenes*: ×, 10 µg/ml oestradiol-17β + 10 µg/ml progesterone; ▲, 10 µg/ml oestradiol-17β + 20 µg/ml progesterone; ■, 20 µg/ml oestradiol-17β + 10 µg/ml progesterone; ○, 20 µg/ml oestradiol-17β + 20 µg/ml progesterone; ●, control 0 µg/ml hormone

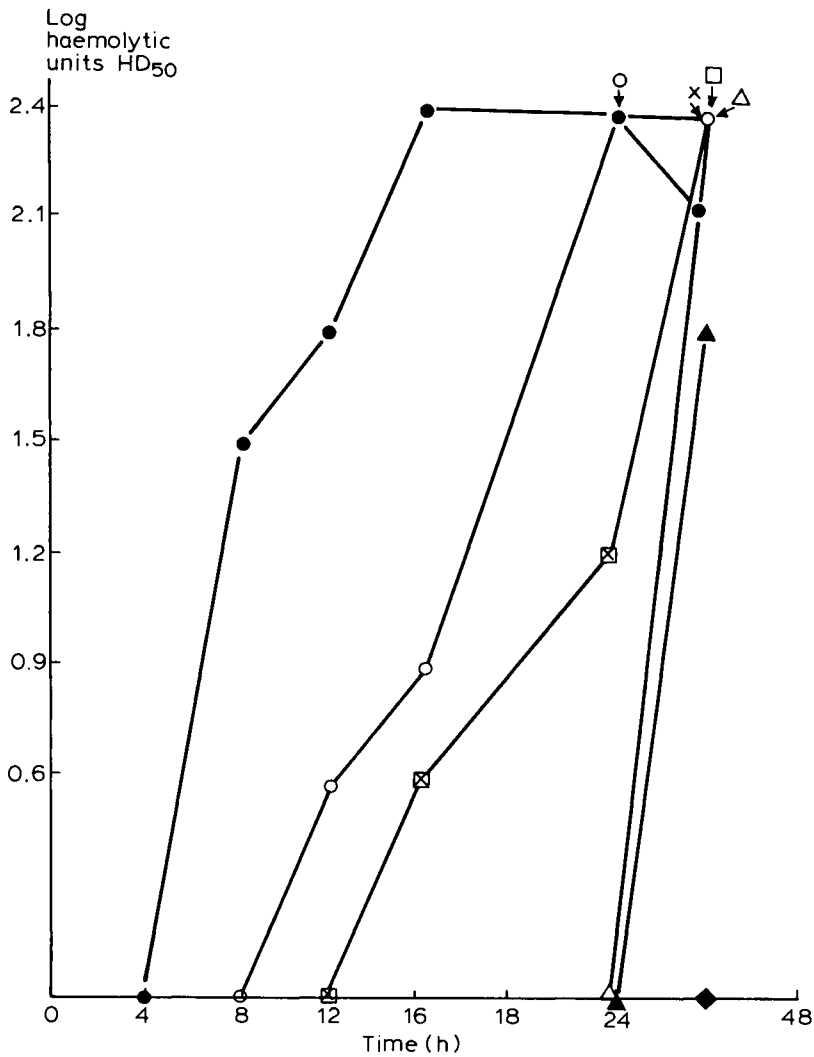


Figure 24.4 Haemolysin production when either progesterone or oestradiol is added to the media. Progesterone (●, control; ○, 10 µg/ml; □, 20 µg/ml; △, 40 µg/ml). Oestradiol-17β (×, 10 µg/ml; ▲, 20 µg/ml; ◆, 40 µg/ml)

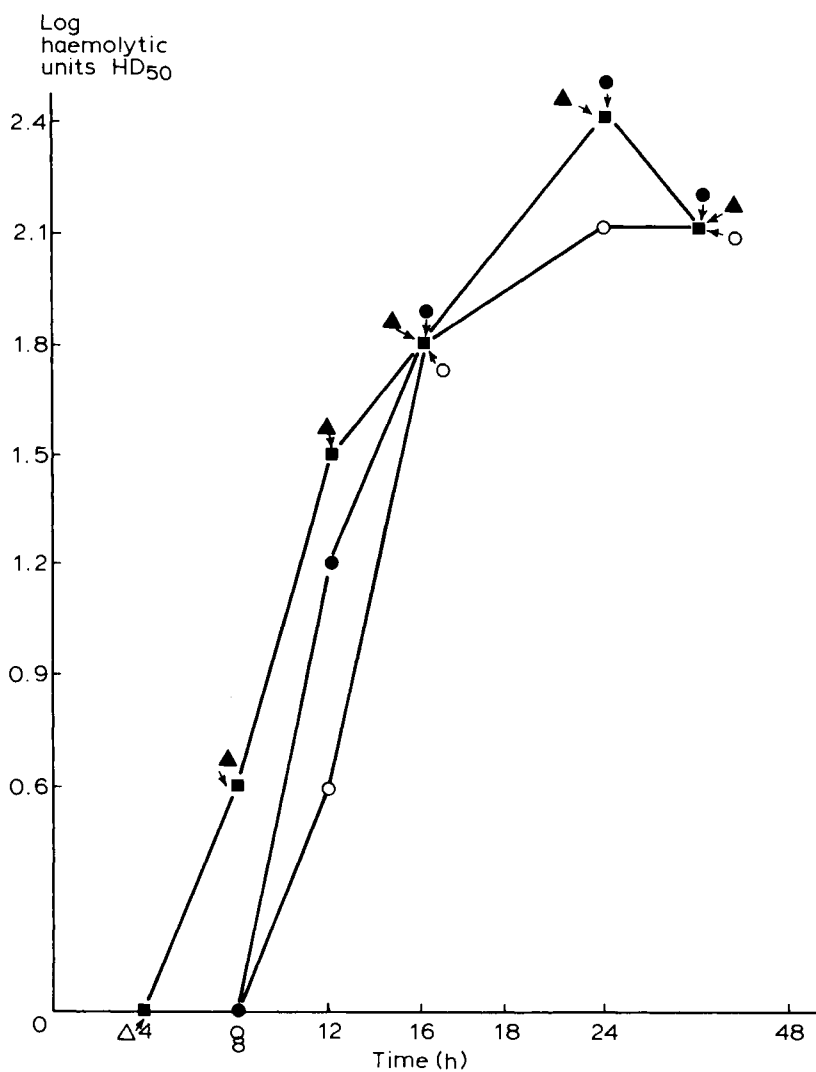


Figure 24.5 Haemolysin production using combinations of oestradiol and progesterone: ■, 10 µg/ml oestradiol-17β + 10 µg/ml progesterone; ○, 20 µg/ml oestradiol-17β + 20 µg/ml progesterone; ▲, 10 µg/ml oestradiol-17β + 20 µg/ml progesterone; ●, 20 µg/ml oestradiol-17β + 10 µg/ml progesterone

gainsay some degree of inhibition occurring during the majority of the hormonal cycle, if both the oestrone derivatives and progesterone individually exert comparative controlling effects on other microorganisms, as they do on both *L. monocytogenes* and its haemolysin.

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PERFORMANCE PROMOTERS IN ANIMAL NUTRITION: II METHODS OF COMPARISON OF EFFECTIVENESS

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Introduction

Performance promotion is a general expression applied to the nutritional responses of apparently healthy animals to the inclusion in the diet of small quantities of medicinal substances, such as antimicrobials and hormones. Antibacterials are extensively used in animal production as prescription-free feed additives to promote the growth of pigs, poultry and cattle. They reduce the unit cost of production via the improvement of feed conversion efficiency and/or rate of liveweight gain.

The performance-promoting activity and the relative effectiveness of different chemicals are neither qualitatively nor quantitatively predictable from molecular constitutions or antibacterial spectra and potencies. Bunyan *et al.* (1977) concluded, from studies on the efficacy of 55 widely varying substances, that growth-promotion properties could not be related with known antimicrobial and absorption characteristics. Several modes of action of performance promoters have been proposed and demonstrated in research laboratories, but their relative contributions in practice are still unknown. Hence, quantification of responses is wholly dependent upon data gathered from controlled feeding tests.

Measured responses in liveweight gain and feed conversion efficiency vary greatly in magnitude with time and place. The assessment of responses and confidence limits, therefore, requires the analysis of many trial results in various environments at different times.

Since Moore *et al.* (1946) first observed a stimulation of chick growth by antibacterials in nutritional studies, a large volume of data has been generated in research and development confirming improvements in animal productivity by more than 50 antibiotics and chemotherapeutics. Generally speaking, data collections of responses have normally been made on a selective basis, and their analysis has been restricted to the calculation of percentage means of control performance. More recent and detailed studies include Fisher (1973), analysing 90 tests on copper sulphate in broilers, Rosen, Roberts and Widowson (1976; 1978) on bacitracin in broilers, Rosen, Roberts and Widowson (1976; 1978) on bacitracin in layers (78 tests) and zinc bacitracin in pigs (221 tests), UKASTA Ltd (1978) on copper in pigs (716 tests) and Rosen (1980b) on zinc bacitracin in broilers (839 tests).

This chapter, which is the second paper of this series, describes a method of comparison of performance promoters based on the detailed factorial analysis of individual antibacterial substances, as reported in the first paper of this series (Rosen, 1980a). The subject matter is chosen to be illustrative and is therefore limited mainly to performance promotion in pigs and to a detailed comparison of virginiamycin and zinc bacitracin. The principles and procedures involved, however, can be similarly applied in current studies on other antibacterials, in various species, to antimicrobial prophylactics, such as coccidiostats, and to therapeutic responses in diseased animals.

Sources of variation in response

Braude, Wallace and Cunha (1953), in an early review, demonstrated that growth-promotion responses vary with dietary antibacterial concentration, animal species, duration of feeding, age of animal, presence of disease, feed specification and level of animal performance. Hays (1981), in a more recent review on the effectiveness of feed additive usage of antibacterial agents in swine and poultry production, includes many tabulated response summaries, revealing manifold differences between maximum and minimum effects with respect to the aforementioned sources of variation. For example, Hays' Table 14 relates the daily weight gain of control pigs to improvements by penicillin plus streptomycin, citing responses of 3.8–27.0% in weight gain and 1.8–11.1% in feed efficiency. Similarly, daily gain and feed efficiency responses to chlortetracycline in pigs are 2.7–10.7% and 4.0–9.2%, respectively, at different protein levels (Table 1) and 10.4–16.7% and 5.1–8.9%, respectively, at different chlortetracycline dietary inclusions (Table 18). Eight different antibacterials manifest 0.7–6.6% and 0.6–3.3% responses in pigs to market weight (Table 27), and responses to chlortetracycline plus sulphamethazine plus penicillin on one farm ranged from 32–93% to 1.0–27.1%, respectively, between 1960 and 1965 (Table 22).

Table 25.1 AVERAGES, COEFFICIENTS OF VARIATION (CoV) AND RANGES OF OBSERVED VALUES FROM SURVEYS OF THE NUTRITIONAL EFFECTS IN PIGS OF TYLOSIN, VIRGINIAMYCIN AND ZINC BACITRACIN ON THE DAILY LIVEWEIGHT GAIN (DLWGEFF) AND FEED CONVERSION RATIO (FCREFF)

No. tests	Substance	Mean dietary concentration (mg/kg)	DLWGEFF (kg/day)			Effects		
			Average	CoV	Range	Average	CoV	Range
Tylosin 477		40.3	0.0460	110	–0.111–0.181	–0.114	147	–0.920–0.740
Virginiamycin 362		26.4	0.0329	116	–0.059–0.186	–0.0987	142	–0.810–0.530
Zinc bacitracin ^a 253		16.1	0.0272	131	–0.074–0.129	–0.0893	222	–1.32–0.430

^a Zinc bacitracin is expressed in mg/kg ($\equiv$ p.p.m.) in this paper, according to the Second International Standard 1 mg $\equiv$ 74 i.u. (Lightbown, Kogut and Uemura, 1964) for comparison with other antibacterials.

Further examples of the extent of variation in response are shown in *Table 25.1*. Nutritional responses are given herein in absolute terms because expression as percentage of control performance does not reveal the potential influence of variation in the latter. The mean response in 477 tests on tylosin in pigs was 0.0460 kg/day, with a range of 0.292 kg/day between limits of an adverse effect of -0.111 kg/day to an improvement of 0.181 kg/day. The mean zinc bacitracin improvement in feed conversion ratio of -0.0893 ranged across 1.75 units of feed conversion ratio, from an improvement of -1.32 to an adverse effect of 0.430. Coefficients of variation of these effects were greater for conversion ratio (142-222%), than for liveweight gain (110-131%).

Appraisals of the effectiveness of performance promoters and comparisons of antibacterials should duly quantify, as far as possible, the influence of key sources of variation.

COMPARISON BY AVERAGE RESPONSE

Lucas (1957) discussed the possibility of comparisons between different antibiotics by bulking data from a large number of experiments testing antibiotic-fed pigs versus corresponding unsupplemented controls. Differences in six such comparisons revealed no clear-cut decision in favour of any one subject, and Lucas criticized this approach because of possible excessive bias from a high proportion of tests on unhealthy or light-weight animals and other inaccuracies of comparison due to variations between experiments in levels of antibiotic fed, in basal diet composition and in the duration of experiments. This threw serious doubt on the suggested superiority of broad- versus narrow-spectrum antibiotics.

Hays' aforementioned review was extensive in citing data from 598 references to journal articles, field-day reports and unpublished data by private communication from researchers for starter, grower-developer and growing-finishing pigs. His summary tables were based mainly on US experiments and data were omitted from studies involving nutrient deficiencies, feed ingredients that exaggerate response and from animals fed individually in metabolism cages. *Table 25.2* contains some examples of his summary results for tests on pigs continued to market weight. From such summaries, Hays concluded that antibacterial agents are obviously not equally effective in improving growth rate and feed efficiency, but his computations were not attempts to compare precisely the relative effectiveness of one drug with another. The presented figures do show differences, but the averaging approach does not necessarily compare like with like. No consideration is given to the influence of the mean levels fed for individual substances or, in some cases, for groupings of different substances designated as arsenicals, tetracycline, etc. No statistical test of the significance of differences is applied, and the influence of differences in mean parameters, ranging between 15-27 kg initial liveweight, 61-94 kg final liveweight, 0.612-0.726 kg average daily gain and 2.96-3.53 feed/gain ratios, is unknown.

Comparisons of valuations, as in *Table 25.2*, solely via the computation of average values of responses, even in absolute terms, would appear to raise more questions than provide answers.

Table 25.2 RESPONSE OF PIGS TO ANTIBIOTICS DURING THE GROWING-FINISHING STAGE (TESTS CONTINUED TO MARKET WEIGHT)

Antibiotic	No. experiments	Liveweight ^a (kg)		ADG ^b (kg)			Feed ÷ Gain		
		I	F	—	+	% ^c	—	+	% ^c
Penicillin-streptomycin	34	22	88	0.703	0.730	3.87	3.44	3.38	1.74
Bacitracin	29	24	93	0.726	0.744	2.50	3.37	3.28	2.67
Tetracycline	108	18	90	0.689	0.735	6.58	3.53	3.44	2.55
Tylosin	26	24	91	0.685	0.717	4.64	3.41	3.36	1.47
Bambermycins	12	27	94	0.721	0.735	1.89	3.42	3.38	1.17
Virginiamycin	21	20	93	0.712	0.753	5.73	3.38	3.27	3.25
Arsenicals	42	15	61	0.612	0.617	0.74	2.96	2.94	0.68
Nitrofurans	7	24	91	0.640	0.649	1.42	3.39	3.37	0.58

Source: after Hays (1981).

^a I, initial; F, final.^b ADG, average daily gain.^c Percentage of improvement.

For example, are the numerically superior responses of virginiamycin over bambermycins, or tetracycline over tylosin, matters of chance; and if not, to what extent are these due to differences in the growth-promoting potency per gram of activity of the different substances, and how far due to the administration of different dietary concentrations? Are the conditions of testing arsenicals and nitrofurans optimal for these substances? What is the influence of test durations inherent in the mean values in *Table 25.2* which range between 75 and 105 days? What bias, if any, is introduced by the use of numbers of experiments from 7 to 108 for different substances?

Even with larger numbers of tests, the problems of simple averaging are evident. *Table 25.3* compares some mean parameters of data banks containing

Table 25.3 COMPARISON OF SOME MEAN CHARACTERISTICS OF DATA BANKS USED IN MULTIFACTORIAL ANALYSES OF NUTRITIONAL RESPONSES IN PIGS FED TYLOSIN (T), VIRGINIAMYCIN (V) AND ZINC BACITRACIN (Z)

Subject	T	V	Z
No of tests	477	362	253
Dietary concentration (mg/kg)	40.3	26.4	16.1
Control liveweight gain (kg)	0.561	0.568	0.607
Control feed conversion ratio (gain ÷ feed)	2.85	2.86	3.18
Duration (days)	59.9	75.1	83.4
Proportion fed <i>ad libitum</i>	0.971	0.862	0.621
Proportion with change in dietary concentration	0.270	0.207	0.198
Year of experiment	1967.9	1972.4	1967.5

477 tylosin tests, 362 virginiamycin and 253 zinc bacitracin in pig feeding. Concerning factors likely to influence response, differences in these subjects for comparison are up to 8% and 12% in gain and conversion control performances, respectively, 39% in duration, 250% in dietary concentration, 56% in feeding system and 36% in discontinuous level of administration.

Comparisons based on average responses only are therefore seen to be of strictly limited value.

COMPARISON BY DIRECT EXPERIMENT

In principle, direct experiment is the correct method of comparison of effectiveness. There are, however, three main drawbacks. First, much larger experiments are needed to determine real differences between responses to two or more promoters, as against the measurement of the magnitude of response of a single subject over a negative control. Secondly, locational and temporal differences in responses, as instanced in *Table 25.1* above, would appear to indicate a need for fairly large-scale replication of already large experiments. And thirdly, the choice of number of levels in the diet of each subject to be fed is problematic. It may not be assumed, indeed the available evidence runs contrary, that the dose-response pattern of each growth promoter is identical in different environments.

Roberts (1983) has calculated the scale of replication for various species required to detect specific differences between treatments for a significance level of 0.05. For pigs from weaning to 90 kg liveweight, having a coefficient of variation in rate of gain of 10% per pig, 50 replicates are needed to detect a 4% difference, with a probability of detection of 0.5, and 100 replicates are

Table 25.4 RESULTS OF COMPARISON OF DAILY LIVELWEIGHT GAIN EFFECTS (DLWGEFF) BY SEVERAL GROWTH PROMOTERS IN THREE DIFFERENT EXPERIMENTS ON PIGS AT VARIOUS DIETARY CONCENTRATIONS (X)

Substance	After Miller et al. (1962)		After Ramage et al. (1963)		After Petersen and Oslage (1974)	
	X (mg/kg)	DLWGEFF (kg/day)	X (mg/kg)	DLWGEFF (kg/day)	X (mg/kg)	DLWGEFF (kg/day)
Carbadox	—	—	—	—	50/0	0.014
Chlortetracycline	22	0.068	22	-0.014	—	—
Oleandomycin	—	—	22	-0.005	20	0
Oxytetracycline	22	0.036	22	0.009	80	0.009
Tylosin	22	0.081	22	0.068 ^a	—	—
Virginiamycin	22	0.072	—	—	50/20	0.040 ^a
Zinc bacitracin	12	0.104 ^a	12	0.005	45/9	0.018
Control daily live-weight gain (kg)	0	0.572	0	0.626	0	0.755
Mean initial live-weight (kg/pig)	—	13.2	—	17.6	—	15
Mean final live-weight (kg/pig)	—	56.6	—	57.0	—	105

^a Significantly better than control.

needed for a probability of detection of 0.8. Even for a lower coefficient of variation of 4%, the corresponding numbers of replicates are 9 and 17, respectively, and 23 replicates would be required for a probability of detection of 0.9.

Some ramifications of problems arising in direct experimental comparisons are illustrated by the data from three experiments summarized in *Table 25.4*. In each experiment only one, each time different, of five antibacterials tested improved daily liveweight gain significantly. Would the use of larger numbers of pigs have revealed significance for the quite substantial performance incre-

ments by other substances? Virginiamycin response in the Petersen and Oslage (1974) trial was also significantly better than the other additives. How would the responses to carbadox and zinc bacitracin have compared if the finisher feed levels had also been 20 mg/kg, or if the test had been terminated at 57 kg liveweight?

The data in *Table 25.5* have been calculated from Langlois, Cromwell and Hays (1978). They show that variations in feed conversion ratio responses in a given environment manifest fluctuations in the comparative pattern of four antibacterials in five replicates conducted at different times during the period 1972–75. Virginiamycin, bacitracin, tylosin or chlortetracycline, virginiamycin and tylosin, successively, gave the largest effect. Virginiamycin afforded the highest mean overall improvement of -0.10 , but individual values fluctuated wildly between a maximum improvement of -0.38 , no response and

Table 25.5 QUINTUPLICATION OF COMPARISON OF FEED CONVERSION RATIO EFFECTS (FCREFF) BY FOUR GROWTH PROMOTERS IN ONE ENVIRONMENT DURING 1972–75 FOR PIGS FROM 14 TO 38 kg LIVEWEIGHT FOR DURATIONS OF 37–53 DAYS

Replicate	FCREFF ^a				Feed conversion ratio of control
	C (44 mg/kg)	B (25 mg/kg)	T (44 mg/kg)	V (44 mg/kg)	
1	+0.01	-0.03	+0.01	-0.25	2.64
2	-0.02	-0.12	-0.09	+0.08	2.39
3	-0.01	+0.05	-0.01	+0.08	2.42
4	-0.21	+0.02	-0.09	-0.38	2.64
5	+0.14	+0.02	-0.02	0	2.44
Range	-0.21–0.14	-0.12–0.05	-0.09–0.01	-0.38–0.08	
Mean	-0.02	-0.02	-0.05	-0.10	
Improvement	3/5	2/5	4/5	2/5	

Source: from Langlois, Cromwell and Hays (1978).

^a C, chlortetracycline; B, bacitracin; T, tylosin; V, virginiamycin.

inferior feed conversion performance in 2 tests of $+0.08$. Subsequent to these results in starter pigs up to 38 kg liveweight, the overall average improvements in feed/gain effects on termination at 98 kg liveweight were chlortetracycline 0, bacitracin -0.06 , tylosin -0.19 and virginiamycin -0.14 . Does the zero outcome for chlortetracycline indicate loss of response since its earlier superior rating over six other antibiotics (Braude, Wallace and Cunha, 1953)? Would an inclusion of bacitracin at 44 mg/kg have equalled the tylosin or virginiamycin responses? And would the differential of tylosin over virginiamycin of -0.05 have been observed over a 4-year period in other environments? Such are the types of question left unanswered by a comprehensive set of trials using 300 pigs.

It is doubtful if any one governmental or industrial organization could aspire to a large-enough scale of replicated comparisons of several performance promoters to measure their relative potencies by direct experiment. In any event, comprehensive assessment of response patterns based on all available data for individual performance promoters might assist the planning and development of more cogent comparisons by direct experiment.

COMPARISON VIA COMPREHENSIVE FACTORIAL ANALYSES OF INDIVIDUAL PERFORMANCE PROMOTERS

Factorial analysis of performance promoter responses consists essentially of the collection and compilation for computer filing of test data from published sources and verifiable personal communications for the elaboration of algebraic models, using standard computer programmes and methods of statistical analysis (Rosen, 1980a). Estimates of response (and confidence limits) for specific conditions of use are calculated from the models for two substances to be compared, and their difference is tested for statistical significance. A detailed comparison of virginiamycin and zinc bacitracin as performance promoters in pigs is used herein to exemplify the method and results.

Table 25.6 MEAN PARAMETERS OF DATA BANKS FOR THE COMPARISON OF VIRGINIAMYCIN (362 TESTS) AND ZINC BACITRACIN (253 TESTS) AS PERFORMANCE PROMOTERS IN PIGS

Parameter	Virginiamycin		Zinc bacitracin	
	mean	s.d. ^a	mean	s.d.
Feed concentration (mg/kg)	26.4	21.3	16.1	17.2
Duration (days)	75.1	34.1	83.1	37.6
Daily liveweight gain effect (kg)	0.0329	0.0381	0.0272	0.0357
Control daily liveweight gain (kg)	0.568	0.138	0.607	0.128
Feed conversion ratio effect	-0.0987	0.140	-0.0893	0.199
Control feed conversion ratio	2.86	0.541	3.18	0.602
Proportion with restricted feeding system	0.138	0.346	0.379	0.486
Proportion with discontinuous feeding concentration	0.207	0.406	0.198	0.399
Year of test	1972.4	6.39	1967.5	6.02

^a s.d., standard deviation.

Table 25.6 sets out the main characteristics of the data banks comprising 362 tests on virginiamycin and 253 on zinc bacitracin. It is evident that the mean gain and conversion responses are similar in magnitude, but differences in mean feed concentration, levels of control performance and proportion utilizing restricted feeding system are evident.

Models obtained to account for the effects of these independent variables on response are given in *Table 25.7*. These regressions account for approximately 20% of the variation in gain and conversion responses. Maximum responses in gain and conversion occur in the range 40–69 mg/kg, with the turning points for conversion improvement at somewhat higher levels than for gain.

Comparison can be made for any desired values of control performance within the confines of the data banks. Responses for 10–70 mg/kg feed concentration have been calculated for pigs from 5 to 95 kg liveweight, gaining 90 kg at a rate of 0.6 kg/day, with a feed conversion ratio of 3.33, involving total feed consumption of 299.7 kg/pig. Estimates of response and 95% confidence limits are shown in *Table 25.8*. At equal inclusion rates in the diet there are no statistically significant differences between the gain or conversion responses by virginiamycin and zinc bacitracin. There are also no statistically

Table 25.7 ALGEBRAIC MODELS FOR EFFECTS OF DAILY LIVEWEIGHT GAIN (DLWGEFF) AND FEED CONVERSION RATIO (FCREFF) OF DIETARY CONCENTRATION (X) OF VIRGINIAMYCIN AND ZINC BACITRACIN; CONTROL PERFORMANCES, DAILY LIVEWEIGHT GAIN OF CONTROL (DLWGC), DAILY FEED INTAKE OF CONTROL (DFIC) AND FEED CONVERSION RATIO OF CONTROL (FCRC); AND FEEDING SYSTEM (DVR = 1 FOR RESTRICTED; = 0 FOR *ad libitum*)

Dependent variable	n ^a	R ^{2b}	S.D. ^c	Constant	X	X ²	DLWGC (kg/day)	DFIC (kg/day)	FCRC	DVR	T.P. ^d (mg/kg)
<i>Virginiamycin</i>											
DLWGEFF (kg/day)	362	0.193	0.0346	0.0362	0.00112 (0.000) ^e	-0.0000106 (0.000)	-0.0833 (0)	—	0.00992 (0.017)	-0.0115 (0.037)	53
FCREFF (mg/kg)	362	0.179	0.128	0.105	-0.00271 (0.008)	0.0000197 (0.046)	0.190 (0.001)	—	-0.0921 (0)	—	69
<i>Zinc bacitracin</i>											
DLWGEFF (kg/day)	253	0.162	0.0330	0.0703	0.000490 (0.083)	-0.00000605 (0.040)	-0.101 (0.001)	+0.0117 (0.098)	—	-0.024 (0)	40
FCREFF (mg/kg)	253	0.240	0.175	0.149	-0.00543 (0.000)	0.0000397 (0.012)	0.512 (0)	—	-0.159 (0)	0.0625 (0.008)	68

^an = number of tests; ^bR = multiple correlation coefficient; ^cS.D., standard deviation; ^dTP, turning point; ^eProbability (in parentheses).

Table 25.8 COMPARISON OF EFFECTS OF VIRGINIAMYCIN (V) AND ZINC BACITRACIN (Z) AT FEED CONCENTRATIONS OF 10–70 mg/kg ON DAILY LIVEWEIGHT GAIN (DLWGEFF) AND FEED CONVERSION RATIO (FCREFF) OF PIGS FROM 5 TO 95 kg LIVEWEIGHT AT 0.6 kg/day DLWGC, 3.33 FCRC and 0.138 DVR

Feed concentration (mg/kg)	DLWGEFF (kg/day)		FCREFF	
	V	Z	V	Z
10	0.028 ± 0.0057 ^a	0.034 ± 0.0050	−0.11 ± 0.021	−0.12 ± 0.027
20	0.036 ± 0.0051	0.037 ± 0.0056	−0.13 ± 0.019	−0.16 ± 0.030
35	0.044 ± 0.0072	0.040 ± 0.0087	−0.16 ± 0.027	−0.21 ± 0.048
50	0.047 ± 0.0090	0.039 ± 0.011	−0.17 ± 0.034	−0.24 ± 0.063
70	0.044 ± 0.011	0.034 ± 0.015	−0.18 ± 0.040	−0.25 ± 0.083

^a 95% confidence limits.

significant differences in the maximal gain and conversion responses. In biological terms there is therefore no difference in effectiveness.

Since performance promoters are used for cost reduction, econometrical comparisons are germane, in view of the demonstrated bioequivalence. Based on a price ratio per gram of activity of virginiamycin to zinc bacitracin of 3.5:1, iso-feed inclusion cost comparisons show that zinc bacitracin affords significantly better improvements in conversion at 70 versus 20 and 35 versus 10 mg/kg, and in gains at 35 versus 10 mg/kg, but not for gains at 70 versus 20 mg/kg.

An econometrical dose-response analysis can be based on the aforementioned pig performance. The total fattening period is 150 days. Mean costs utilized are feed at £160/t, virginiamycin at £0.060/g activity and zinc bacitracin at £0.017/g activity. Overhead costs are calculated as one-sixth of the cost of feed, and savings therein are proportional to the number of days saved from 5 to 95 kg slaughter weight. Net profit per pig is calculated as the sum of savings in feed and overheads, less the cost of the amount of growth promoter used.

Table 25.9 reveals maximum economic benefits from virginiamycin and

Table 25.9 NET MARGIN/PIG DUE TO FEED AND OVERHEADS COST REDUCTION BY VIRGINIAMYCIN (V) AND ZINC BACITRACIN (Z) AT FEED CONCENTRATIONS OF 10–70 mg/kg

Feed concentration (mg/kg)	Net margin (£/pig)	
	V	Z
10	1.80 (10) ^a	2.07 (40)
20	2.03 (5.9)	2.65 (27)
43	2.24 (3.1)	—
50	2.22 (2.6)	3.68 (15)
64	—	3.76 (12)
70	1.95 (1.6)	3.73 (11)
Feed	£160/t	
V	£0.06/g activity	
Z	£0.017/g activity	

^a Net margin ÷ Cost of growth promoter (in parentheses).

zinc bacitracin at dietary inclusion concentrations of 43 and 64 mg/kg, respectively, amounting to £2.24 and £3.76/pig. The advantage of £1.52/pig for zinc bacitracin stems mainly from its greater feed conversion improvement and its lower cost price. Net margin/growth promoter cost ratios for zinc bacitracin are substantially higher than those for virginiamycin, the importance of which relates to the large variations in response about the mean which occur from time to time.

A similar econometrical pattern has been found in the comparison of virginiamycin and zinc bacitracin for broilers, based on comprehensive studies of 326 tests on the former (Rosen, 1983), and 839 tests on the latter (Rosen, 1980b). Maximum net margins in broilers are found at dietary concentrations of 58 and 64 mg/kg of £29.8 and £41.5/1000 birds, respectively, for virginiamycin and zinc bacitracin.

Further studies are in progress on the factorial evaluation of responses of pigs to avoparcin, olaquindox and tylosin, and broilers to avoparcin and nitrovin, to extend the comparisons of biological and econometrical effectiveness along the lines exemplified herein.

Conclusion

Liveweight gain and feed conversion responses to the nutritional use of antibacterials as performance promoters vary considerably with factors such as dietary concentration, level of control of animal performance and feeding system. This variation precludes the use of average responses for the comparison of effectiveness of different antibacterials.

Comparison by direct experiment is severely limited by the need for very large-scale, highly replicated trial programmes capable of measurement of small significant differences.

Multifactorial analysis of large collections of responses to individual antibacterials has been used as a basis for biological and economic comparisons of effectiveness.

A detailed comparison of virginiamycin and zinc bacitracin as performance promoters in pigs reveals their biological equivalence and the economic advantage of zinc bacitracin.

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CONTEMPORARY CONCEPTS OF GROWTH PROMOTER USE

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Introduction

It is well known that feed additives with antibacterial activities are more efficient as growth-promoting agents than substances without those properties. This may be explained by the fact that they act not only as anabolic agents (Schole, Harrisch and Salmann, 1978), but possess also nutrient sparing and metabolic effects, mediated by changes in absorption or in the microflora of the alimentary tract (Coates, 1980; O'Connor, 1980). There is evidence that there is a close correlation between the antibacterial spectrum of a compound and the growth promotant effect it produces: the broader this spectrum of activity, the greater the weight increase and feed conversion improvement and the lower rates of mortality and morbidity per unit of time (Gedek, 1974; 1979a; 1979b). This emphasizes the importance of use of antibacterials as growth promoters under the intensive conditions of animal production on farms in highly industrialized countries, where the animals are stressed by factors such as overcrowding, temperature variation, subclinical disease, suboptimal nutrition, mixing and transport (Walton, 1983). Thus, the most obvious application for growth promoters in farm animals is in creep, starter and grower diets.

On the other hand, the beneficial effect of modifying the metabolic activity of the obligate gut flora and the consequent reduction in toxin production may be at the risk of the development of resistant organisms? The disappearance of the nutritive effect after prolonged administration of a feed additive has repeatedly been attributed to this (Menke and Krampitz, 1972). Since, however, this effect has been mainly observed with the use of substances which are also used therapeutically in animals, it seems doubtful whether this phenomenon can be observed when the compound is more frequently used in 'sub-therapeutic doses'; this remains a subject of discussion (Linton, 1977).

Possible theoretical hazards

Over many years there has been a considerable bias, particularly by the medical profession, an attitude acquired avidly by consumerists, against the

use of antimicrobial substances in sub-therapeutic doses to enhance animal production. This antagonism has been expressed particularly towards the use of agents which also have a utility in human therapeutics. These attitudes became formalized in the Swann Report of 1969, which recommended *inter alia* that antibiotics used for human therapy should not be used for growth-promotion purposes in view of the then imagined dangers of the induction of bacterial resistance. We can now list three main imagined dangers, which can now realistically be regarded as misconceptions, as follows:

- (1) The use of antimicrobial agents at sub-therapeutic levels could select bacterial strains resistant to that agent.
- (2) The continuing high prevalence of bacterial resistance in farm animal populations was a direct result of using sub-therapeutic levels in feeding-stuffs.
- (3) Bacterial resistance to antibiotics, especially transmissible antibiotic resistance in salmonellae, would rapidly spread from farm animals to man, again under the influence of using sub-therapeutic levels of antibiotics in animal feeds.

The previous practice of applying the same antibiotic for both therapeutic and nutritional purposes made the quantification of the influence of the latter on the development of resistant bacteria difficult.

Meanwhile, several of the new alternative substances, which follow, with the exception of some macrolides, the example set by flavophospholipol and virginiamycin in not being used for treatment of animals, have already been used for some years, and as the first effects of withdrawal in the EEC member states of tetracyclines in animal production are already measurable, it is now appropriate to make an assessment of the situation.

Effects on resistance patterns of the growth-promotional and therapeutic usage of antimicrobials in farm animals

The inefficiency of the Swann Report's recommendation to ban sub-therapeutic use of tetracyclines in animal production was amply illustrated by Jackson (1981). He showed that the percentage of resistant strains among *Escherichia coli* isolates from farm animals in the UK during the years 1971-77 was substantially unchanged, despite the ban on sub-therapeutic usage of tetracyclines in animal production. In contrast to the expected decrease of resistance to tetracyclines since that time, there is some tendency to observe that *E. coli* isolates from farm animals have developed more and more multiple resistance to drugs (Gedek, 1981a; 1981b).

In Germany it was determined, from faecal examinations, that calves increasingly excreted *E. coli* bacteria with a simultaneous resistance to five antibiotics and also sulphonamides (*Table 26.1*) (Gedek, 1972; Gedek and Schael, 1976). The problems associated with intensive farming of veal calves often require repeated use of 'medicated' feed containing prescription-only medicaments both for prophylaxis and for treatment, not least because the presently permitted feed additives, with their spectrum limited to Gram-positive organisms, are no substitutes for the tetracyclines (Gedek, 1979a). In

Table 26.1 INCIDENCE OF RESISTANCE TO FIVE DIFFERENT ANTIBIOTICS AND SULPHONAMIDES IN *Escherichia coli* ($n = 3840$) FROM THE FAECAL FLORA OF VEAL CALVES ($n = 384$) AFTER ADMINISTRATION OF MEDICATED FEEDS AND SUBSEQUENT APPLICATION OF GROWTH PROMOTERS DURING 10 WEEKS^a — RESULTS OBTAINED UNDER IDENTICAL CONDITIONS AT INTERVALS OF THREE YEARS

Chemotherapeutic	Resistance 1973		Resistance 1976		Resistance 1979		Resistance 1982	
	(rates, %)		(rates, %)		(rates, %)		(rates, %)	
	Medicated feed	Growth promoter	Medicated feed	Growth promoter	Medicated feed	Growth promoter	Medicated feed	Growth promoter
Tetracycline-HCl	99.3	87.4	100	93.6	99.8	80.6	96.7	86.2
Streptomycin	97.8	92.8	100	93.6	94.4	72.3	95.8	82.3
Chloramphenicol	91.2	65.8	98.9	39.8	79.7	47.5	47.8	6.5
Ampicillin	89.0	53.9	43.8	12.8	87.3	29.8	47.9	6.2
Kanamycin	74.3	3.0	97.9	44.7	56.6	18.0	49.2	8.0
Sulphonamides	97.8	89.8	100	91.5	100	96.2	87.6	81.3

^a Antibiotics as growth promoters were Zn-bacitracin, flavophospholipol, avoparcin and others.

this animal species, a different bacterial resistance situation must result as a consequence of the higher selective pressure caused by therapy. A parallel is found in the intensively farmed chicken (Gedek, 1980).

In pigs we had experienced, over many years, a better situation due to the widespread routine use, during the first period of fattening (Gedek, 1979b), of chemobiotics such as carbadox and olaquinox. The latter are effective against Gram-negative organisms and not only reduce the number of *E. coli* bacteria, but by a special effect on the DNA of the bacteria cell acting mechanism, they also actively eliminate R-factors. Data from feeding experiments carried out in England are in agreement with results first obtained in Germany (Gedek, 1979a) and are statistically significant (Davey, 1979). Therefore, it was possible that the extensive use of chemically derived antibacterials in pig feed has contributed to the more favourable resistance situation among the porcine intestinal bacteria in Germany (Table 26.2). But this situation was drastically changed by the decision to use these chemobiotics together with copper sulphate at high levels (up to 200 p.p.m. Cu^{2+}) in pig feeds (Gedek, 1981a). This was due to the fact that EEC Directive 70/524 has prohibited only the simultaneous use of carbadox and olaquinox with antibiotics, but not with copper salts for growth promotion. Now we have, to almost the same extent as in calves, a high incidence of *E. coli* strains from pigs showing multiple resistance (Table 26.1 and 26.2).

In this connection, the use of various substances with antibacterial activity for different purposes (which involves not just antibiotics since there are also chemical compounds) deserves consideration. There is now evidence that the prevalence of multiple drug resistant enteric bacteria such as *E. coli*, and the emergence of special types of resistance patterns, are closely related to the increasing use, in the intensive farming of pigs and other animals, of combinations of several drugs in 'medicated feed' for the treatment of infectious diseases and prophylaxis, instead of a single antibiotic. In pigs, it was stated that under practical conditions, sulphonamides were very strong selectors of multiple resistance to five antibiotics (given in Table 26.1), and this plasmid-mediated type of 4-6-fold multiple resistance in *E. coli* is now predominant in all farm animals after therapeutic treatment (Gedek, 1981a; 1981b; 1981c). Combinations of antibiotics are more powerful selective agents than the individual antibiotics when used alone. This was demonstrated several years ago with chlortetracycline alone, in comparison to a combination of chlortetracycline, dihydrostreptomycin and penicillin G, added to feed to protect calves against infectious diseases (Gedek, 1980). The tests, in calves known to have *E. coli* with 5-6-fold drug resistance patterns in their intestines, have shown that a treatment with a high dose of chlortetracycline as a therapeutic did not result in an increase of such multiple resistant bacteria, whereas the latter usually become prevalent after use of a combination of several drugs, mainly used in feed to treat and prevent diseases, as was done in the experiment (Gedek, 1980).

Very similar results were obtained recently when sodium arsanilate was given alone to pigs in drinking water, in comparison to a combination in feed containing, in addition to sodium arsanilate, sulphonamides and furazolidone (Table 26.3). While the latter select more for *E. coli* with 5-6-fold drug resistance patterns, strains of the same species with 2-3-fold drug resistance patterns became predominant after treatment with the single drug (Table

Table 26.2 INCIDENCE OF RESISTANCE TO FIVE DIFFERENT ANTIBIOTICS AND SULPHONAMIDES IN *Escherichia coli* ($n = 2560$) FROM THE FAECAL FLORA OF PIGS ($n = 640$): A COMPARISON BETWEEN THE THERAPEUTIC AND NUTRITIONAL USE OF SUBSTANCES WITH ANTIBACTERIAL ACTIVITY AT VARIOUS TIMES

Chemotherapeutic	Medicated feed		Feed chemobiotics		Feed antibiotics	
	Antibiotics in combination with chemicals	(<i>E. coli</i> resistant, %)	Carbadox alone	Carbadox + copper sulphate	Tylosin, Zn-bacitracin and others	(<i>E. coli</i> resistant, %)
	1977-78	1980-81	1977-78	1980-81	1977-78	1980-81
Tetracycline-HCl	71.5	97.3	68.2	55.1	86.7	77.1
Streptomycin	75.7	87.2	38.5	78.8	63.4	77.0
Chloramphenicol	38.6	66.0	2.5	34.3	12.3	9.5
Ampicillin	34.6	45.9	4.1	28.6	14.4	14.5
Kanamycin	13.8	13.9	0	6.1	0.9	0.8
Sulphonamides	98.6	92.9	31.8	60.8	84.8	81.1
No. farms	6	6	5	5	5	5
No. animals	120	120	100	100	100	100

Table 26.3 FREQUENCY OF SINGLE AND MULTIPLE RESISTANCE TO CERTAIN CHEMOTHERAPEUTICS IN *Escherichia coli* ($n=690$) FROM THE FAECAL FLORA OF PIGS ($n=150$) TREATED WITH SODIUM ARSANILATE—A COMPARISON BETWEEN USE IN MEDICATED FEED AND AS SINGLE DRUG IN DRINKING WATER

	Sodium arsanilate combined with sulphonamide and furazolidone in feed (%)		Sodium arsanilate without any other drug in drinking water (%)	
	After 1 week medicated feed		After 10 weeks feeding Zn-bacitracin	
<i>Resistance</i>				
Sixfold	11.6	0.8	0	1.6
Fivefold	37.9	6.0	9.2	60.8
Fourfold	27.3	20.0	20.0	20.0
Threefold	17.4	40.8	3.6	6.8
Twofold	5.3	24.4		
Single	0.5			
<i>Sensitivity</i>				
to all 6 chemotherapeutics tested ^a	0	4.4	1.6	
$\bar{x}$ number of resistance determinants per isolate	4.3	2.9	2.7	
No. isolates	190	250	250	
No. animals	50	50	50	

^a Tetracycline-HCl, streptomycin, chloramphenicol, ampicillin, kanamycin, sulphonamides.

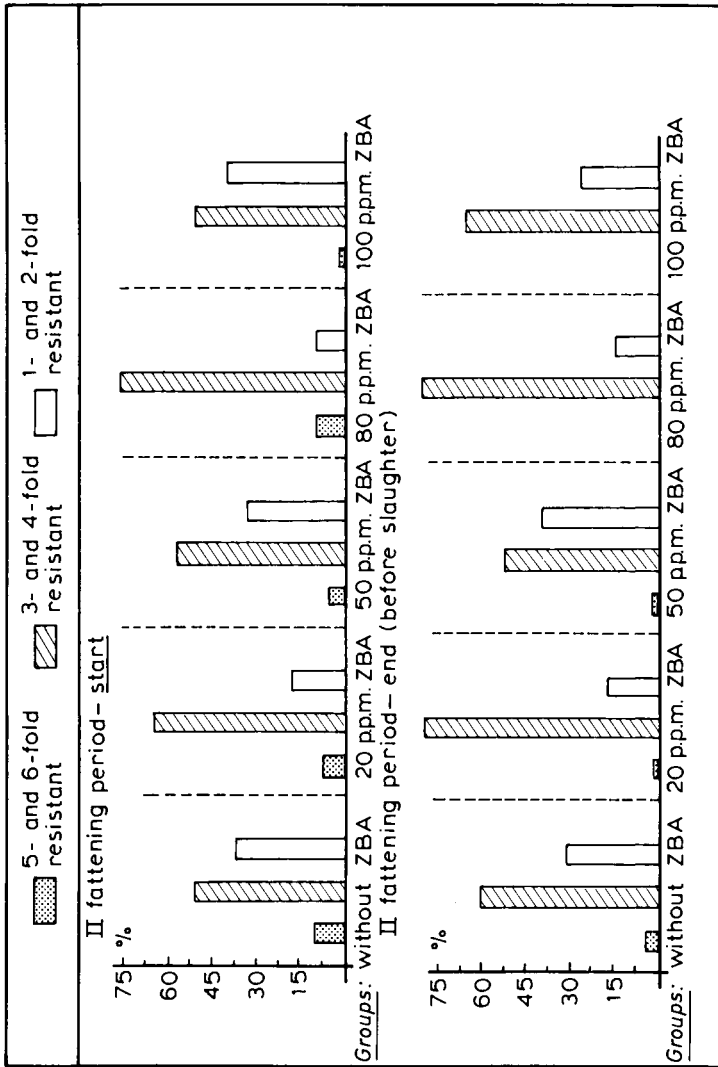


Figure 26.1 Frequency of single and multiple resistance to certain chemotherapeutics^a in *Escherichia coli* from the faecal flora of pigs (n = 100)^b after administration of sodium arsenite in drinking water

^a Tetracycline-HCl, streptomycin, chloramphenicol, ampicillin, kanamycin, sulphonamides.

^b Two trials with Zn-bacitracin (ZBA) as growth promoter — 10 pigs per group.

26.3). The selection of strains which showed resistance to streptomycin more frequently than to tetracyclines and other drugs was observed at any time, and was not influenced by 'medicated feed' use before or during growth-promoter application at different levels (*Figure 26.1*, e.g. zinc-bacitracin). With copper sulphate at a lower level, such as 150 p.p.m. Cu^{2+} in pig feed, very similar results were obtained, but at a higher level such as 200 p.p.m. Cu^{2+} it appeared that copper sulphate must, like sulphonamides,

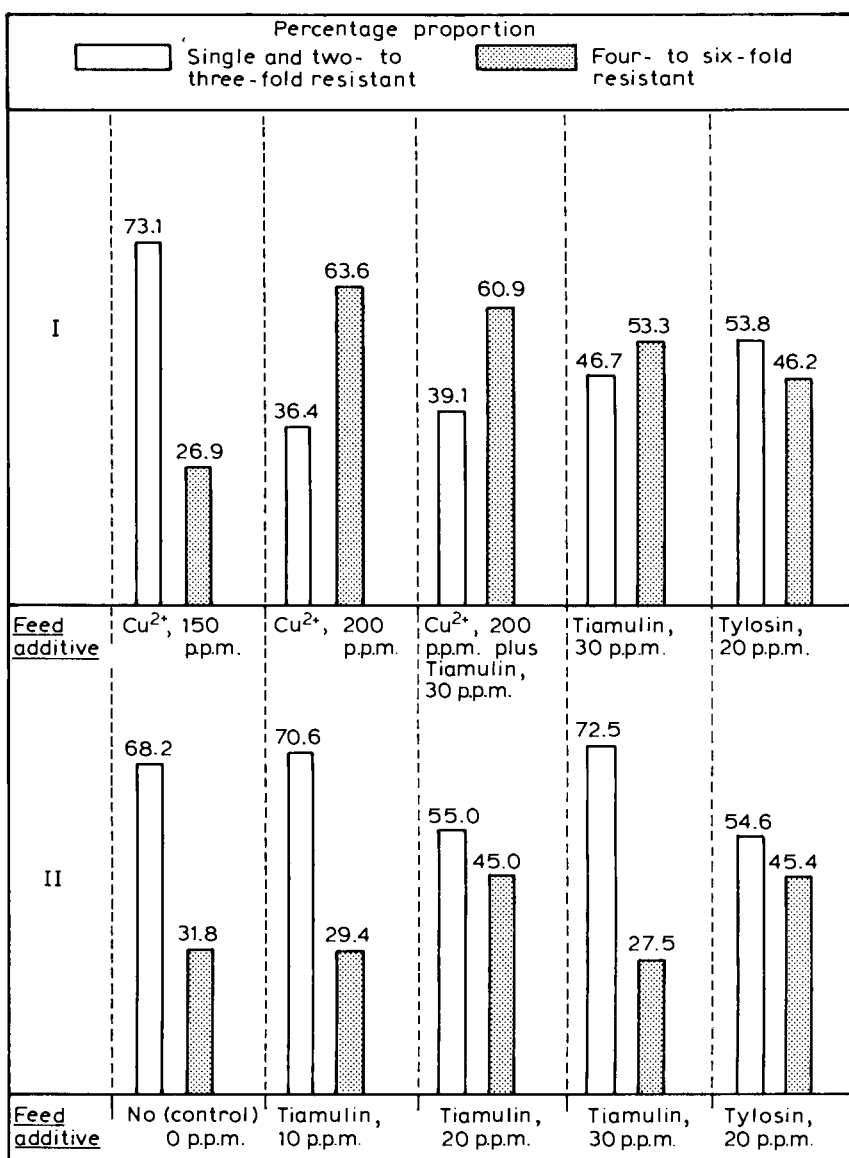


Figure 26.2 Occurrence of drug-resistant *Escherichia coli* in the faecal flora of pigs after 9-10 weeks' application of the growth promoters—trials I and II

be a strong selector of higher multiple resistant *E. coli* strains (Figure 26.2). With use of 200 p.p.m. Cu^{2+} in the diet, *E. coli* 4–6-fold multiple drug resistant strains became predominant, whereas 2–3-fold or single drug resistant strains became prevalent with 150 p.p.m. Cu^{2+} in the diet (Figure 26.2). Contrary to these findings with other substances used at growth-promoter levels, we obtained detrimental results, e.g. also with tiamulin, when added at levels up to 30 p.p.m. in pig feed (Figure 26.2).

To date, it has been possible to demonstrate that, during the use of each new antibiotic growth promoter, there is a gradual decrease in the number of R-factor-carrying bacteria in the faecal flora of animals at slaughter, provided the simultaneous use of high level therapeutics is avoided. This can be ascribed to the fact that in the state of eubiosis, R-plasmid-carrying bacteria such as *E. coli* and other enteric bacteria, like enterococci and staphylococci, are fewer in number (Figure 26.3) than those types of bacteria in which the

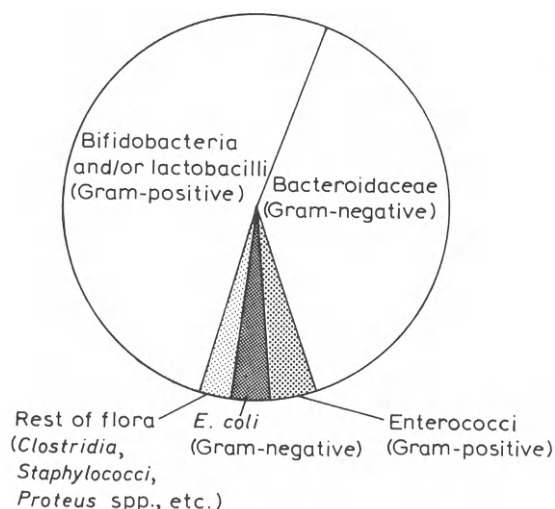


Figure 26.3 Composition of the intestinal flora of livestock in the state of eubiosis (shaded areas, part of the bacteria with proven transferable chemoresistance)

process of gene transfer is not relevant (Anderson, 1974), and to the fact that gene transfer is to a great extent hindered by metabolites of these bacteria (Wiedemann, 1972). Thus, the transmission of genetic information during conjugation remains at a normal level in *E. coli* (Jarolmen and Kemp, 1969).

Provided that the biological equilibrium of the intestine is maintained, one may observe in the R-plasmid-carrying types, during the stationary phase of their development, vegetative segregation of extrachromosomal genetic material, whereby transfer factor and resistance characteristics are lost, together with other coded characteristics, e.g. colicin production (Gedek and Schael, 1976). Such a spontaneous loss was first observed in *E. coli* in the faecal flora of calves concerning resistance to chloramphenicol, kanamycin/neomycin and ampicillin as well as to tetracyclines (Gedek and Schael, 1976), and was confirmed also for Gram-positive organisms of the gut flora of farm animals, such as staphylococci (Gedek, 1978; Figures 26.4 and 26.5).

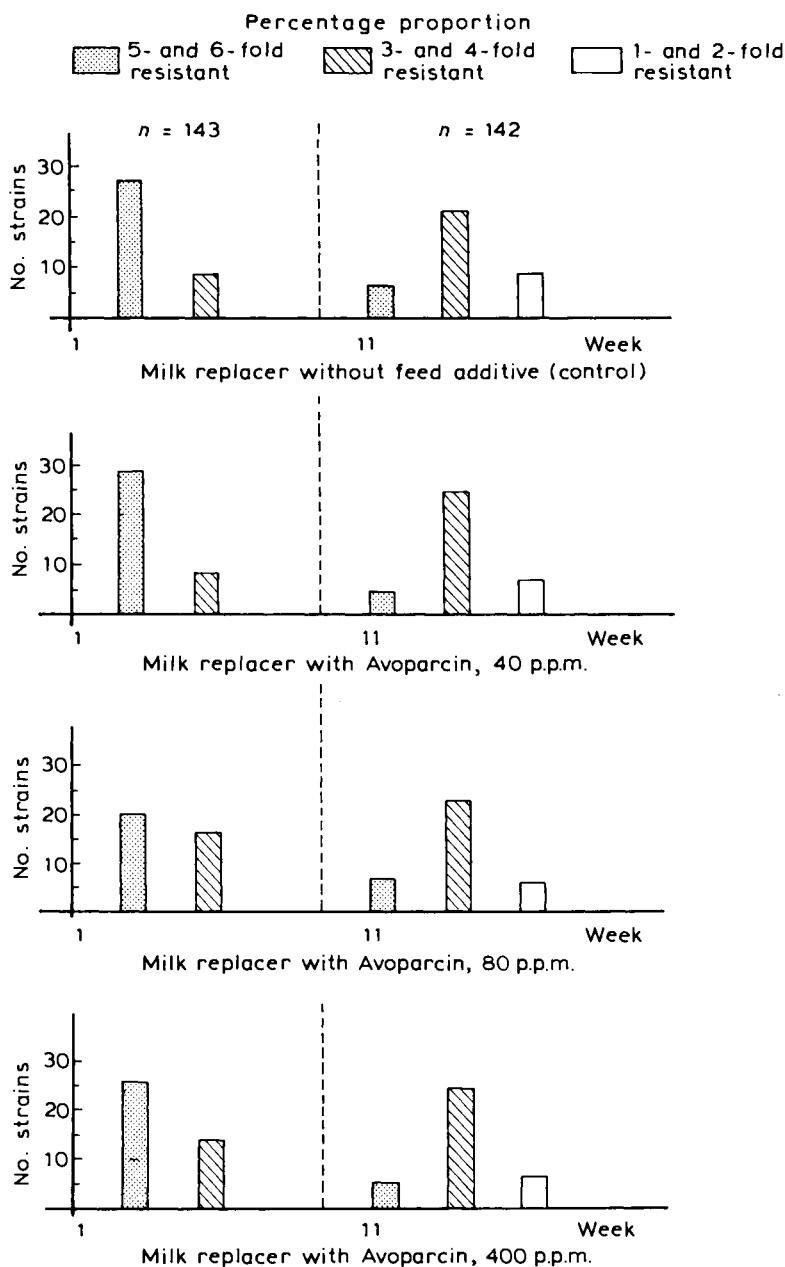


Figure 26.4 Occurrence of drug-resistant *Escherichia coli* in the faecal flora of veal calves at the start and end of the feeding experiment ($n = 36$) (chemotherapeutics tested were: tetracycline-HCl, streptomycin, chloramphenicol, ampicillin, kanamycin, sulphonamides)

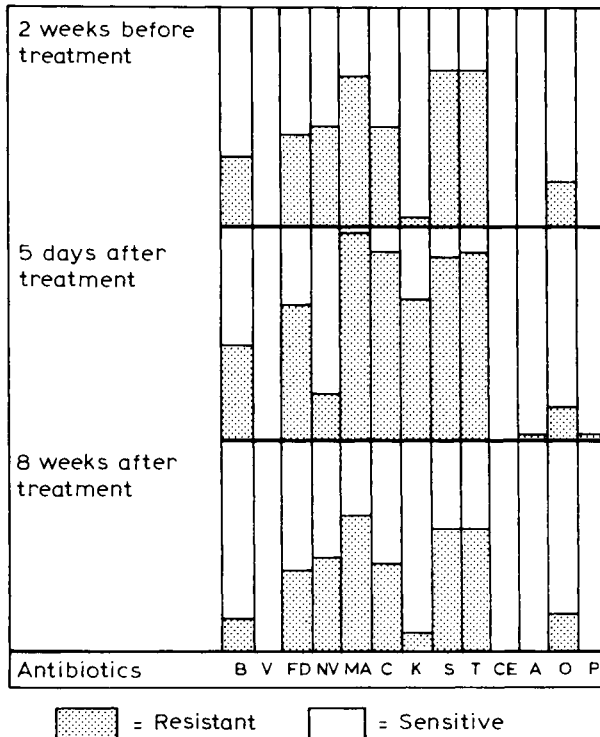


Figure 26.5 Resistance pattern of staphylococci isolated from faeces of calves ($n=48$) before and after administration of medicated feed (P, penicillin-G; O, oxacillin; A, ampicillin; CE, cephalothin; T, tetracycline; S, streptomycin; K, kanamycin/neomycin; C, chloramphenicol; MA, macrolides; NV, novobiocin; FD, fusidic acid; V, virginiamycin; B, bacitracin)

From the different resistance patterns recorded, it appears that the portion of resistant bacteria decreases, in general (Levine, 1980), when an antibiotic at growth-promoter levels has been used over a period of several weeks. Because, among other things, the nutritive effect of a substance with antibacterial activity implies that, when added in small quantities to the feed, it stabilizes the intestinal flora (Visek, 1978), it is not surprising that, even when using a new type of growth promoter, the resistant bacteria present in the gut flora of an animal do not disappear at once (Gedek, 1979b).

Presently, plasmids are so widespread that one has always to anticipate their appearance (Mitsuhashi, Rosival and Kramery, 1977). For this reason R-plasmids are encountered in the intestinal flora of humans and animal, even in the absence of recent antibiotic treatment (Wiedemann, 1974; Fagerberg *et al.*, 1978; Gedek, 1980, 1981b; Richmond, 1981). Some individuals eliminate the same R-factors in their faeces over weeks and months, whereas in others there is some variation in the different types of R-factor-carrying *E. coli* in their faecal flora (Siegel, Huber and Enloe, 1974; Gedek and Schael, 1976; Langlois, Cromwell and Hays, 1978a; 1978b). In ignorance of this fact, workers have repeatedly misinterpreted the significance of the persistence of *E. coli* bacteria with multiple drug resistance following the use of new types

of growth promoters (Linton, Howe and Osborne, 1975; Linton, 1977; Hirsh and Wiger, 1978).

For each new growth promoter, it can be stated that a reduction in bacterial sensitivity after its use in farm animals is entirely due to chromosome-related resistance (Gedek, 1980). Usually, however, bacteria modified by mutation appear to be less pathogenic and do not constitute an environmental hazard. Their longer generation time precludes their development in competition with the faster growing types prevalent in nature, and the antibiotics which might give them, *in vivo*, a selective advantage, are quickly destroyed by the soil microflora (Jagnow, 1977).

Even so, it can be demonstrated that bacteria having such a chromosomal resistance are not simultaneously resistant to another chemotherapeutic having the same mechanism of action, but belonging to another group of substances (Gedek, 1980). Thus, I believe, the updated evidence now indicates that the earlier fears concerning sub-therapeutic usage of antibiotics and other imagined deleterious effects on human health were unfounded, and the Swann Report's policy of a rigid distinction between 'feed' and 'therapeutic' use of antibiotics was unnecessary. The emergence of plasmid-mediated resistance to antibiotics is dependent upon the use of drugs which are selectors of this type of resistance. The possibility that properties other than antibiotic resistance may also be selected cannot be excluded, if they are on the same plasmid or coexistent in another plasmid in the same bacterium, or even located within the chromosome. In spite of the fact that plasmid-mediated resistance is now widely distributed, each separate ecosystem shows a considerable variation in the type and frequency of resistance, particularly to drugs; thus there cannot exist a permanent exchange of genes between bio-types of bacteria belonging to the microflora of man and animals.

A rationale for the future use of antimicrobials in animal feeds

The economies of countries where modern, intensive animal production is practised cannot successfully accept any further interference with their competitiveness (Gilliam and Martin, 1975; National Academy of Sciences, 1980). As Professor M.H. Richmond has stated (Richmond, 1981), 'one must stress that antibiotics have properties which make them valuable for a wide range of purposes many of them far from the formally therapeutic'.

A decision against the modern growth promoters in favour of the exclusive use of 'medicated feeds', consisting always of combinations of different high level therapeutic medicaments, under veterinary prescription, would aggravate the resistance situation and not attain the required result. It is possible that, by then, the growth promoters would no longer possess the capacity to stimulate higher production and, in certain cases, to preclude the need for the use of the therapeutics which latter we know to be responsible for the selection of multiple drug resistant bacteria.

So far as the growth promoters are concerned, every attempt to increase the levels of use to therapeutic levels should be resisted; otherwise the positive effects of low level usage experienced to date will be reversed. Instead, in order to maintain the efficiency of these substances, it would be worth while investigating the value of extending the antibacterial spectrum of the presently

permitted narrow spectrum growth-promoting antibiotics, by combining these antibiotics with other growth-promotant agents, such as enzymes or selected strains of lactic acid bacteria (*Lactobacillus* spp. or *Streptococcus faecium*), which have a general non-specific action on metabolism, in addition to preventing diseases associated with the early rearing/growing phases of life.

One of the essential factors in industrialized animal production is the need to provide feed adjuvants which (1) prevent endemic disease; (2) improve growth rate and feed conversion efficiency at an economic cost; (3) promote better utilization of nutrients, and (4) reduce the need for therapeutic interventions. The latter interventions carry the attendant risks of tissue residue problems, in addition to the problem of R-plasmid prevalence which is aggravated by therapeutic treatment. An increased agricultural efficiency, with respect to both growth promotion and elimination of R-factors, could be achieved through the use of antimicrobial feed adjuvants and lead to fundamental economic benefits without hazard to host animal, consumers or the environment.

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ANTIBIOTICS AS FEED ADDITIVES FOR RUMINANT LIVESTOCK

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Introduction

During the past two decades there has been a very considerable expansion of intensive livestock production in many of the developed countries of the world. There is little reason to doubt that this expansion will continue in the reasonably foreseeable future, nor that such expansion has made, and will continue to make, a major contribution to the production of high quality protein for human consumption. Antibiotics have played a considerable role in enabling such intensification to be successful. Indeed, although their use in agriculture has many objectives, as is highlighted in various chapters of this book, it is their use in the food animal industry that is of most importance.

With reference to the livestock industry, antibiotics have three roles to play. First, many antibiotics perform a vital role as therapeutic agents in the treatment of disease. Thus, in the treatment of mastitis, intramammary formulations of the penicillins, penicillin G and cloxacillin, of the amino glycoside streptomycin, of the macrolide erythromycin and of the tetracyclines, oxytetracycline and chlortetracycline have all proved valuable.

Their second role is in the treatment of healthy animals to contain the spread of infection, i.e. disease prevention or prophylaxis.

Their third role is one characterized by the use of dosage rates which are low in comparison with those used therapeutically; continual administration of antibiotics in feed at such low dosage rates, to animals that are considered clinically and nutritionally normal, results in improved animal performance in terms of growth rate and/or feed conversion efficiency.

It is this last-mentioned usage that brings certain antibiotics into the group of substances generally termed feed additives. It is very likely that a number of these antibiotics, when used at the low levels associated with improvements in animal performance among healthy normal animals, are also acting to maintain the health of the animals in the face of environmental stress and the challenges that are an inevitable consequence of modern systems of intensive livestock production, and to this extent they are acting prophylactically.

Not surprisingly, in view of the antimicrobial nature of these products, their early use as feed additives was almost exclusively directed towards pigs, poultry and the non-ruminant veal calf; the dependence of ruminating ani-

mals such as cattle and sheep upon an active and flourishing microbial biomass in their forestomachs to initiate the digestive process was well recognized. However, with the introduction of certain of the more recent classes of antibiotic, notably the ionophores, the situation has changed significantly. Specific antibiotics are now widely and successfully used as feed additives for growth promotion, particularly in beef cattle. It is the intention, in this chapter, to detail the role of antibiotics as feed additives in ruminant livestock and to refer to what is known concerning their mode of action.

The term antibiotic has tended to become synonymous with 'antibacterial'. However, a number of available antibiotics possess activity against a variety of other living organisms, including viruses, fungi, protozoa and helminths (Hudd, 1983). This point is relevant to any consideration of the effect of feed antibiotics in ruminant livestock, since it is now recognized that, in addition to bacteria and protozoa, the microbial biomass within the rumen contains a sizeable component of fungi (Bauchop, 1979).

Legislation

The fear in the 1960s that continued use of low levels of antibiotics as growth promoters in simple-stomached animals might lead to the development and selection of bacteria that were resistant to the effects of these, or other, related antibiotics led to the introduction of legislation in the UK—the Medicines Act 1968—which classified antibiotics under two headings: Therapeutic and Feed. Subsequently, legislation has been passed in the EEC, Directive 70/524, 1970, on Feed Additives, which allocates antibiotics and other antimicrobials to one of three categories: Annexe I, Annexe II, and prescription only products; the last-mentioned cannot be used as feed additives. Regulations governing the use of antibiotics are the subject of Chapter 37, and so little further reference will be made here to this important aspect.

EEC feed additive legislation also implies that the use of antibiotic feed additives should not leave 'unacceptable' traces of antibiotic or its residues in meat, milk and eggs and has established maximum permitted residues. Thus, as Hudd (1983) points out, there is a tendency towards using as feed additives those antibiotics that are not absorbed from the alimentary tract at the low dosages used or, if so, are only poorly absorbed, although in the latter case the need for a withdrawal period before slaughter makes the product less acceptable to the livestock industry. The molecular size of the antibiotic may be of significance with reference to the extent of its absorption from the digestive tract. Thus, the antibiotics avoparcin, bacitracin and flavomycin have molecular weights in excess of 1000 (Hudd, 1983), and according to this last-mentioned author there are no reports of residues appearing in the tissues even when fed at high levels. On the other hand, the tetracyclines, with molecular weights approximating to 500, leave detectable residues when given at nutritional levels (Hudd, 1983). The tetracyclines, as important therapeutic antibiotics in human and veterinary medicine are, of course, now banned from use as feed additives in the EEC.

Nature and efficacy of antibiotics for growth promotion in ruminant livestock

Up to the present, the first and most successful antibiotic used as a feed additive for ruminant livestock belongs to a group of antibacterials termed ionophores, which essentially are lipophilic compounds capable of rendering cations lipid soluble by binding them to their surface. The earliest of these compounds was monensin (called rumensin in the UK); more recently, studies have been undertaken, or are in progress, on another three ionophores—lasalocid, narasin and salinomycin. All were initially selected as effective coccidiostats for poultry.

Ionophore antibiotics

MONENSIN SODIUM ($C_{36}H_{61}O_{11}Na$; mol. wt 692)

This is a polyether antibiotic synthesized by *Streptomyces cinnamonensis*, from which it derives its name. It has a special affinity for Na^+ and this facilitates its entry into the cell, in addition to altering the activity of the Na^+/K^+ pump in the pericytoplasmic membrane (Austic and Smith, 1980).

Studies in steers have shown that over 70% of monensin is excreted as the unchanged antibiotic in the faeces and that in cattle receiving up to 500 mg/day per head, no residues were detectable in body tissues at zero withdrawal time, using an assay with a sensitivity of 5 ng/kg (Hudd, 1983). Traces of the product did appear in the liver of cattle receiving daily doses of 750 mg of the product (i.e. more than three times the maximum authorized level) for 106 days before slaughter, but were not present following a 2-day withdrawal period (EEC, 1980). The Scientific Committee for Animal Nutrition have recommended (EEC, 1980) that the maximum amount of monensin sodium in the daily ration of beef cattle should not exceed the level given by the following relationship: maximum daily dose = $80 \text{ mg} + 60 \text{ mg}/100 \text{ kg}$ live-weight. Translating such data for varying liveweights into mg/kg dry matter complete feedingstuff gives values ranging from 35 to 40.

In indoor-fed beef cattle, the effectiveness of inclusion of a low level of monensin in feed—the recommended level is 30 mg/kg feed—can be reflected in an increased rate of gain with no change in feed intake (Dinius *et al.*, 1978; Poos, Hanson and Klopfenstein, 1979) or alternatively no change in daily rate of gain but a significant reduction in daily feed intake (Mowat, Wilton and Buchanan-Smith, 1977; Pendlum, Boling and Bradley, 1978; Bartley *et al.*, 1979; Johnson *et al.*, 1979; Byers, 1980). Part of the variability in response is undoubtedly related to factors such as differences in ration formulation and level of dietary crude protein. Thus, Hanson and Klopfenstein (1979) showed that response to monensin was greater on a low protein than on a high protein diet. However, it is noteworthy that increased efficiency of feed conversion occurs on high roughage diets (Bartley *et al.*, 1979) as well as on high concentrate (energy) rations (Heinemann, Hanks and Young, 1978).

The nature and magnitude of the response to low-level inclusion of monensin in rations for cattle is also dependent upon the level of inclusion of the antibiotic. Figure 27.1(a) shows the change in mean daily liveweight gains and mean daily feed intakes in beef cattle fed a high energy ration (corn 41–70%;

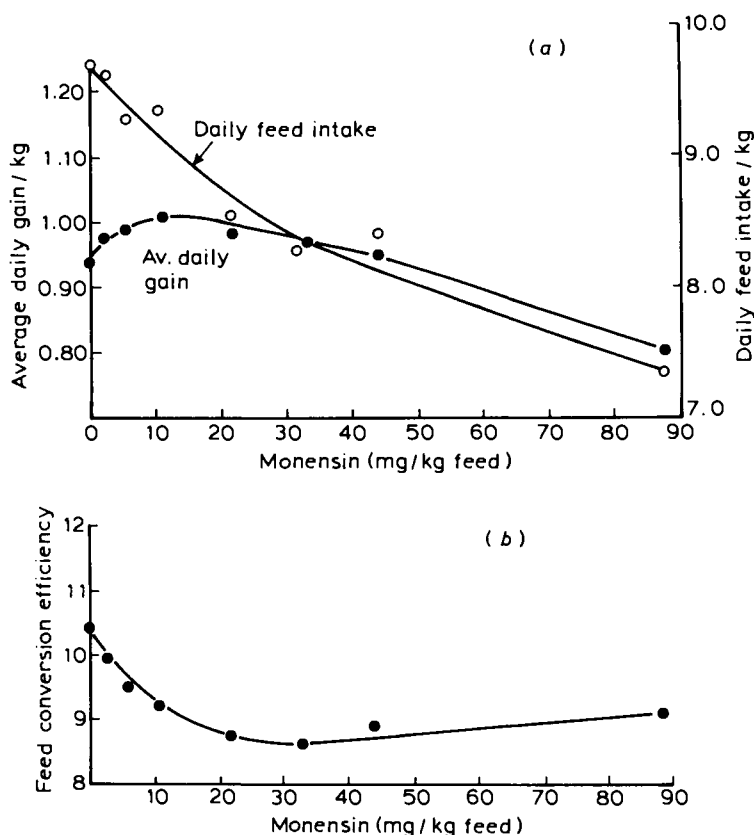


Figure 27.1 (a) Mean daily liveweight gains and mean daily feed intakes in beef cattle fed varying levels of monensin as a feed additive; (b) feed conversion efficiency in beef cattle fed varying levels of monensin as a feed additive (from Raun *et al.*, 1976, by courtesy of the American Society of Animal Science, publisher of the *Journal of Animal Science*)

corn cobs 10–35%; soya bean meal 8–9%; lucerne meal 3–5%) containing levels of monensin ranging from zero to 88 mg/kg feed (Raun *et al.*, 1976). It can be seen that at levels of inclusion of 50 mg/kg feed and below, daily liveweight gain tended to equal or be superior to that of control livestock; higher levels depressed daily rate of gain. Feed intake was depressed at all levels of inclusion. *Figure 27.1(b)* shows that feed conversion efficiency was always greater at all levels of inclusion of monensin, but tended to be maximal at 30–35 mg/kg. At this level of inclusion, liveweight gain was some 3.2% greater, feed intake 14.4% lower and feed conversion efficiency 17.1% higher than for the cattle fed the non-medicated feed.

The above-mentioned data relate to North American studies. Hawkrig (1980) has analysed the results of some 35 trials carried out in 9 countries in Europe involving beef cattle fed monensin-supplemented diets, and the data are summarized in *Table 27.1*. From *Table 27.1* it can be seen that at dose levels of 25–33 mg/kg, average daily gain was some 5.2% greater than that of control animals and was associated with a 4.0% lower feed intake; overall

improvement in feed conversion efficiency at this level of inclusion approximated to 8.7%.

Wilkinson *et al.* (1980) have summarized the results of 12 trials involving beef cattle maintained under pasture conditions and given either 200 mg monensin/head per day in 0.5–1.0 kg of a carrier supplement, or a similar amount of supplement with no antibiotic inclusion. The mean duration of the trials was 119 days and the overall mean daily liveweight gains of the control and monensin-treated livestock were 0.786 and 0.893 kg/head per day. The 13.7% difference in favour of the cattle receiving the medicated supplement was highly significant ($P < 0.001$) and showed no tendency to decline as the trials proceeded. Trials in the USA (Males, Hunt and Lee, 1979) showed that cattle wintered on dry, mature pasture and receiving

Table 27.1 THE SUMMARIZED RESULTS OF 35 TRIALS CARRIED OUT IN EUROPE ON THE EFFECT OF MONENSIN AS A FEED ADDITIVE FOR BEEF CATTLE

Monensin	No. of replicates	Average daily gain (kg)	Average daily feed (kg)	Feed conversion ratio
0	94	1.153	7.45	6.59
10–13	11	1.206 (4.60)*	7.28 (–2.28)	6.16 (–6.53)
16–21	14	1.196 (3.73)	7.19 (–3.76)	6.05 (–8.19)
25–33	77	1.213 (5.20)	7.15 (–4.03)	6.02 (–8.65)
37–40	16	1.208 (4.77)	6.95 (–6.71)	5.91 (–10.32)

Source: from Hawkridge (1980).

*Values in parentheses indicate percentage change from control.

200 mg monensin/head per day in a supplement gained 12.3% faster (0.64 v. 0.57 kg/day) than animals on the same pasture given the non-medicated supplement; the difference was significant ($P < 0.05$). In connection with pasture studies, a convenient way of administering the antibiotic is to incorporate it into a feed block; an alternative is to administer it in a controlled-release capsule placed in the rumen (Watson and Laby, 1978).

In an experiment with growing/fattening bulls given monensin at 33 mg/kg feed, Daenicke, Rohr and Oslage (1982) observed a 4.8% increase in daily gain, with no change in feed intake, resulting in a 5.1% better feed conversion efficiency. Carcass composition studies showed that energy retention was some 15–19% higher in bulls receiving the medicated feed compared to controls; there was no change in protein content, but fat content increased by 15.3%.

Responses to the inclusion of monensin in lamb-fattening trials have also been reported. Thus, Joyner *et al.* (1979) observed that inclusions of the antibiotic at 20 and 30 mg/kg in a low-energy diet reduced feed intake over a 60-day period by 6.4% and 18.4%, respectively, compared to the intake of non-medicated feed, although weight gains were not affected; improvements in feed conversion values were 8% and 13%, respectively. Similar trends in growth improvement of lambs, i.e. no change in rate of gain but reduction in feed intake, were observed by Horton and Stockdale (1981) when feed containing 22 mg monensin/kg was fed; compared with controls mean daily feed intake was reduced by 7.1% and feed conversion efficiency improved by 8.8%. In the studies of Horton (1980), lambs fed a high barley ration containing

33 mg/kg monensin gained 8.1% faster than lambs fed the non-medicated ration with no difference in mean daily feed intake; feed conversion efficiency on the medicated feed was improved by 5.7%.

LASALOCID SODIUM ($C_{34}H_{53}O_8Na$; mol. wt 612)

This is another polyether antibiotic, which is, like monensin, an ionophore and like monensin is used as an anticoccidial agent in poultry.

In experiments with Holstein heifers, Bartley *et al.* (1979) observed that at an inclusion rate of 33 mg/kg feed, the antibiotic had a very similar effect to monensin, i.e. no significant effect on weight gain compared with controls, but a reduction in feed intake (12%), which resulted in a marked improvement (14%) in feed conversion efficiency. Horton and Stockdale (1981), in a 103-day growth trial with early weaned, male crossbred lambs carrying a heavy natural infection of coccidia, observed that the inclusion of lasalocid in feed at levels ranging from 12.5 to 100 mg/kg feed increased weight gains over controls by 6% ($P < 0.05$); feed conversion efficiency averaged 9% higher for lambs receiving the medicated feed at levels of 25, 50 and 100 mg of the antibiotic/kg feed; at the 12.5 mg level of inclusion, feed conversion efficiency was 7.5% higher than in controls.

SALINOMYCIN ($C_{42}H_{69}O_{11}Na$; mol. wt 772)

This polyether antibiotic is produced by fermentation of *S. albus* (ATCC 21838) isolated from a soil in Japan. It is only effective against Gram-positive bacteria and not against Gram-negative organisms, fungi or helminths. It is essentially a monocarboxylic acid containing five cyclic ether rings and like the other ionophores was initially developed as a coccidiostat for poultry, turkeys and rabbits.

It is also used as a growth promoter in lambs and fattening cattle at levels in feed ranging from 10 to 30 mg/kg feed as well as in pigs (25–50 mg/kg feed). Like the other ionophores, it is not used in human medicine.

From the results of some 18 trials with beef cattle involving more than 1000 animals, levels of salinomycin between 100 and 200 mg/head per day gave 7–11% improvement in daily liveweight gain and some 12% improvement in feed conversion efficiency. In experiments with lambs, incorporation levels of 10–30 mg/kg complete feed resulted in weight gains approximating to 10%; these were associated with 17% increases in feed conversion efficiency (P. Elias, personal communication). In view of small tissue residues detectable in liver and bile of chickens and mice given the product, the Scientific Committee on Animal Nutrition (EEC, 1980) recommends a 5-day withdrawal period for use with this antibiotic. The same is likely to apply to lasalocid and narasin.

NARASIN ($C_{43}H_{71}O_{11}Na$; mol. wt 784)

This polyether antibiotic is produced by fermentation of a strain of *S. aureofaciens* and, apart from the addition of a methyl group in place of a hydrogen,

it has the same structure as salinomycin. Again this ionophore is a potent anticoccidial; it has activity against some Gram-positive anaerobes, but is less active against mycoplasma and Gram-negative anaerobes and is inactive against Gram-negative aerobes. It would seem likely that in the future it may find a role comparable to other ionophores as a feed additive for ruminant livestock.

Other antibiotics

AVOPARCIN ($C_{53}H_{60}O_{30}N_6Cl_3$; mol. wt 1312)

This glycopeptide antibiotic of known structure is produced from fermentation of a strain of *S. candidus* and is active against Gram-positive bacteria. It is used solely as a feed additive. The growth-promoting effects of avoparcin for pigs and poultry are well established.

In recent years, studies with cattle have shown that the antibiotic has growth-promoting properties in cattle. Johnson *et al.* (1979) studied the effect of including 16.5, 33 and 66 mg/kg complete feed in feedlot cattle and, as can be seen from Table 27.2, the two higher levels of inclusion increased daily gain

Table 27.2 EFFECT OF AVOPARCIN ON PERFORMANCE IN FEEDLOT CATTLE OVER A 112-DAY PERIOD

	0	Avoparcin (mg/kg feed)		
		16.5	33	66
Average daily gain (kg)	1.18 ^{a*}	1.19 ^a (101)†	1.27 ^b (108)	1.28 ^b (109)
Average daily feed intake (kg)	10.55	10.37 (98)	10.62 (101)	10.54 (100)
Feed conversion efficiency	8.97 ^a	8.71 ^{ab} (97)	8.38 ^{bc} (93)	8.19 ^c (91)

Source: from Johnson *et al.* (1979), by courtesy of the American Society of Animal Science, publisher of the *Journal of Animal Science*.

*Means on same line with different superscripts differ significantly ($P < 0.05$).

†Values in parentheses are values as percentage of controls.

and improved feed conversion efficiency. In the experiments of Dyer *et al.* (1980) with yearling heifers fed for 140 days on a high barley diet, inclusion of avoparcin as a feed additive at 33, 49.5 or 66 mg/kg complete feed gave no increases in daily liveweight gain over control animals fed the non-medicated feed; with all three levels of inclusion, feed conversion efficiency was significantly improved compared with that for the control animals. Mudd and Smith (1982) have summarized the results of some 30 trials carried out in Europe and the USA on the use of avoparcin as a growth promoter in beef cattle. Avoparcin levels ranged from 15 to 60 mg/kg feed intake and for animals receiving the antibiotic for 130 days or longer, mean improvements in daily liveweight gain and in feed:gain ratio were 5.4% and 10.6%, respectively. Reference has already been made to the fact that there is no evidence of absorption of the antibiotic from the ruminant's digestive tract.

FLAVOPHOSPHOLIPOL

This is a glycolipid antibiotic produced by the fermentation of *S. bambergiensis*. It is frequently termed bambermycin and is commonly used under the name flavomycin. It is effective against Gram-positive microorganisms and is not absorbed to any significant extent from the digestive tract when used as a feed additive for fattening cattle. The Scientific Committee for Animal Nutrition has recommended (EEC, 1980) that, for use as a feed additive in cattle, the maximum amount of flavophospholipol in the daily ration should not exceed that given by the following relationship: $25 \text{ mg} + 15 \text{ mg}/100 \text{ kg}$ liveweight. Converting intakes calculated for various liveweights according to the equation gives maximum concentrations of flavophospholipol to be permitted in feeds for beef cattle of 9–12 mg/kg complete feedingstuff.

VIRGINIAMYCIN

This narrow spectrum antibiotic, acting primarily against Gram-positive microorganisms, is produced by the fermentation of a strain of *S. virginiae* originally isolated from a soil in Belgium. It is a natural mixture of two distinct chemical components, one of which, a peptolide, has a molecular weight of 525 and the other, a macrocyclic lactone, has a molecular weight of 823 (Biot, 1979; Hudd, 1983). Virginiamycin has been used in human medicine for the treatment of staphylococcal infections and in veterinary medicine for the treatment of swine dysentery. Parigi-Bini (1979) has reported, in an 18-week growth trial with steers fed the antibiotic at levels of either 25 or 50 mg/kg feed, that mean daily growth rate was significantly ($P < 0.05$) increased with either level of antibiotic by 6% compared with animals receiving the non-medicated feed. Compared with the results from the control animals, mean daily feed intakes were increased by 5% and 2%, respectively, while feed conversion efficiencies were 99% and 96% of those for the controls; none of these differences was statistically significant. Limited absorption of the antibiotic does occur from the alimentary tract (O'Connor, 1980).

TYLOSIN

This is a macrolide antibiotic produced by the fermentation of a strain of *S. fradiae* obtained from a soil sample from Thailand (Hudd, 1983). It has a molecular weight of 903. It is mainly active against Gram-positive bacteria and mycoplasmas and is used in veterinary medicine for the control of respiratory infections in chickens, turkeys, pigs and cattle. It is not used in human medicine.

When the antibiotic was fed continuously to feedlot cattle at 75 mg/head per day (Brown *et al.*, 1975), or at 11 mg/kg complete feed (Heinemann, Hanks and Young, 1978), there was no evidence of any significant growth-promoting effect, although in both trials the inclusion of tylosin markedly reduced the incidence and severity of liver abscesses. This was also observed by Horton and Nicholson (1980) in cattle receiving 11 mg tylosin/kg complete feed. According to O'Connor (1980), tylosin is capable of being absorbed

from the alimentary tract, although only at levels above those used nutritionally (Hudd, 1983).

THIOPEPTIN

This narrow spectrum, Gram-negative antibacterial is a sulphur-containing peptide antibiotic which is known to be very effective against *Streptococcus bovis* (Muir and Barrets, 1979). While steers receiving the antibiotic at 11 or 22 mg/kg feed initially showed a marked improvement in liveweight gain compared to controls, by 4 weeks there was no evidence of any growth-promotion characteristics (Muir *et al.*, 1981). However, it has been shown to be effective in preventing lactic acidosis in sheep following intra-ruminal administration of ground wheat (Muir *et al.*, 1980). In the studies of Muir *et al.* (1981), referred to above, rumen lactate levels were, respectively, 80% and 70% less than in non-medicated controls and the presence of the antibiotic appeared to allow 'normal' fermentation to proceed. Thiopeptin is not absorbed from the alimentary tract (O'Connor, 1980).

Mode of action of growth-promoting antibiotics

THE MECHANISM OF ACTION OF THE ANTIBIOTICS

Of the ionophore antibiotics, most is known about valinomycin which has a special affinity for K^+ . Like all ionophores, it forms a lipophilic complex with K^+ ions and the complex can then pass from the inner face of the cytoplasmic membrane to the outer face where the ion is discharged. The valinomycin molecule then returns to the inner side of the membrane to pick up another ion. The net effect is to drain the cell of K^+ , thus preventing energy production and protein synthesis leading to death of the cell (Hammond and Lambert, 1978). As already mentioned, monensin has a special affinity for Na^+ and facilitates its entry into the cell, thereby altering the activity of the Na^+/K^+ pump. It is noteworthy that ionophores are not used clinically since they are equally toxic to mammalian and bacterial cells (Hammond and Lambert, 1978).

Avoparcin interferes with bacterial cell wall biosynthesis through its prevention of the formation of the cell wall component peptidoglycan (Speth, Greenstein and Maiese, 1981). Flavophospholipol (and bacitracin and the penicillins) act similarly, although not necessarily each by the same mechanism. Since it is the Gram-positive organisms which have peptidoglycans in their cell wall, it is not surprising that the above-mentioned antibiotics are mainly effective against Gram-positive organisms. Nevertheless, it is noteworthy that several species of bacteria, e.g. *Butyrivibrio fibrisolvens* and rumenococci species, which routinely stain Gram-negative in the laboratory have, when viewed under the electron microscope, a Gram-positive ultrastructure and not surprisingly respond to antibiotics such as avoparcin in a similar way to Gram-positive organisms; such organisms are physiologically Gram-positive (M.V. Crossley, personal communication).

The macrolide antibiotic tylosin (and others of this class, namely spiro-

mycin, oleandomycin and erythromycin) effectively inhibits bacterial protein synthesis within the cell, as does virginiamycin, the tetracyclines, the peptide antibiotic thiopeptin and the lincosamide antibiotic lincomycin, although again the method of inhibition differs between antibiotics. Virginiamycin inhibits protein synthesis in the bacterial cell at the ribosome level by preventing the formation of the peptide bonds (Biot, 1979).

MODE OF ACTION WITH RESPECT TO GROWTH PROMOTION

As Coates (1980) points out, the most convincing evidence that the mode of action of the antibiotics is associated with their antibacterial action lies in the demonstration that the growth of germ-free chicks is not improved by including antibiotics in the diet—in contrast to the effect that such antibiotics have on their conventional counterparts. In the case of the ruminant animal, it is to be expected that, provided the antibiotic or some proportion of it survives the rumen environment, then the overall effect of the antibiotic as a feed additive is likely to be a composite of the effect on the micro-flora and -fauna of the reticulo-rumen and that arising from any effect of the antibiotic in the small intestine, and indeed quite possibly in the caecum and colon also. In sheep fed a pelleted diet containing 45 mg/kg avoparcin, microbiological assay revealed that appreciable amounts of the antibiotic were present in the digesta entering and leaving the small intestine (Macgregor and Armstrong, 1982).

In view of the foregoing it is proposed to consider, first, events occurring within the reticulo-rumen and subsequently the significance of the antibiotic in the small intestine. The growth-promoting action of antibiotics and the suggestions that have been made concerning their mode of action has been recently reviewed by Visek (1978), Coates (1980) and O'Connor (1980) and, with particular reference to the ruminant animal, by Broome (1980) and Macgregor (1983).

EVENTS WITHIN THE RUMEN

One of the most consistent features associated with the presence of antibiotics in the feed of ruminants is an increase in molar proportion of propionate at the expense of acetate and frequently butyrate (Mowat, Wilton and Buchanan-Smith, 1977; Ingle, Dalrymple and Kiernan, 1978; Prange, Davis and Clark, 1978; Daenicke, Rohr and Oslage, 1982). Associated with this change in volatile fatty acid proportions in rumen liquor, is a decline in methane production. With monensin, the production of methane is reduced by 30–40% (J.D. Allen, D.G. Harrison and D.G. Armstrong, unpublished observations).

Using isotope dilution techniques, Van Maanen *et al.* (1978) reported that, in cattle receiving 150 mg monensin/head per day, rumen propionate production was increased from 510 to 899 g/day on a high concentrate diet and from 441 to 695 g/day on a high roughage diet. Similarly, Prange, Davis and Clark (1978) observed an increase in propionate production from 572 to 827 g/day in cattle fed a hay/concentrate diet when given feed containing 33 mg monen-

sin/kg. In sheep receiving 22 mg monensin/kg feed, Allen and Harrison (1979) observed that propionate production increased by 32.6% (from 0.95 to 1.26 mol/day) and the change was accompanied by a small (5.3%) increase in acetate (2.83–2.98 mol/day) and a considerable decline (35.2%) in butyrate (0.366–0.129 mol/day); total moles of volatile fatty acid produced/day in the rumen increased from 4.21 to 4.55 in the presence of the ionophore.

Enhanced propionate production can be achieved when ruminants are fed on cereal-based diets (Orskov, Fraser and Gordon, 1974). In sheep, in extreme circumstances, the propionate thus produced may serve directly as a precursor of odd-numbered fatty acids or indirectly, via its metabolite, methyl malonate, as a precursor of branched chain fatty acids (Scaife and Garton, 1975; Scaife, Wahle and Garton, 1978). It has been noted that when sheep and cattle are fed on diets which produce a high concentration of rumen propionate, sheep accumulate branched-chain fatty acids whereas cattle do not produce any significant amounts of these unusual fatty acids (Duncan and Garton, 1978) which give rise to soft fats. It has recently been shown in lambs (Livesey, 1979) that a combination of cereal-rich diet and monensin supplementation can provide propionate concentrations high enough to produce significant quantities of odd-numbered and branched chain fatty acids.

Henderson, Stewart and Nekrep (1981), in agreement with the findings of Chen and Wolin (1979), have shown, in studies of monensin on pure cultures of rumen bacteria, that the increase in propionate is likely to result from the selective inhibition of the growth of rumen bacteria which are not important producers of propionate, thus favouring growth of organisms like *Anaerovibrio lipolytica*, *Selenomonas ruminantium*, *Megasphaera elsdenii* and *Bacterioides ruminicola* which produce propionate. Ruminococcus species and *B. fibrisolvens* are major acetate and hydrogen producers in the rumen and depression of their growth by monensin would decrease yields of these products. In this connection, it is noteworthy that Henderson, Stewart and Nekrep (1981) observed that monensin had only a slightly inhibiting effect on *Methanobacterium ruminantium* and thus it would appear that the reduction in methane arises mainly from a shortage of hydrogen rather than extensive inhibition of a major methane-producing bacterium. This finding is in agreement with that of Van Nevel and Demeyer (1977).

Monensin has also been shown to affect nitrogen metabolism in the rumen. Poos, Hanson and Klopfenstein (1979) with cattle and Allen and Harrison (1979) with sheep, have shown that in the presence of monensin there is a decreased flow of microbial protein into the small intestine although, at least in the studies of Poos, Hanson and Klopfenstein (1979), the decreased flow of microbial protein was associated with increased entry of feed protein into the small intestine, the net effect being little change in total nitrogen entry into the small intestine. When consideration is given to the considerable level of nucleic acid nitrogen in microbial biomass, overall amino acid supply to the host animal would be likely to increase. In connection with the lower microbial yield in the presence of monensin, it is interesting to note that Henderson, Stewart and Nekrep (1981) observed that, in the presence of 10 µg monensin/ml, there were smaller yields of bacteria/mol of substrate fermented.

Broome (1980) has summarized the effects of monensin on apparent digestibility in sheep and cattle and has concluded that, while the effects on organic matter are slight and rarely significant, those relating to nitrogen are positive

and, although small, may be real. Little significance can be attached to values for overall nitrogen digestibility in ruminants (Lindsay and Armstrong, 1982). The apparent increase in nitrogen digestibility may reflect decreasing excretion in the faeces of microbial cell matter, arising in part from the decreased flow of microbial biomass into the small intestine, and in part from decreased microbial biomass production in the caecum and colon due to the presence of the antibiotic therein.

The presence of monensin decreases rumen solids and liquid turnover rates (Lemenager *et al.*, 1978; Allen and Harrison, 1979). The last-mentioned workers observed that decreased dilution rate associated with monensin feeding was reflected in an increase in the amount of organic matter fermented in the rumen. The combined effect of a selection for propionate producers among the microbial population, increased organic matter digestion in the rumen and decreased methane production would give rise to an increased intake of metabolizable energy per unit of feed dry matter. Evidence that this is so can be seen in the results of Rowe, Davies and Broome (1982), who observed an increase of 20% in metabolizable energy intake when monensin was included in the feed. Daenicke, Rohr and Oslage (1982), from their detailed studies on growing bulls, concluded that the presence of monensin in the feed increased efficiency of energy utilization. They considered that part, at least, of this improved efficiency resulted from an increased metabolizability of the feed energy, which was not associated with increase in overall digestibility but with the enhanced propionate production and reduced methane production. Certainly, if the metabolizability of the feed energy increases, there would be expected to be an increase in efficiency of use of the metabolizable energy (ARC, 1980). A further factor may well be the increase in proportion of energy deposited as fat rather than protein (Armstrong, 1982).

Chen and Wolin (1979) showed that lasalocid, like monensin, favoured propionate-producing species of bacteria at the expense of those which give rise to acetate and hydrogen. M. V. Crossley (personal communication), in a comparable study to that of Henderson, Stewart and Nekrep (1981) but using avoparcin, has shown that the antibiotic is very similar to the ionophore in favouring propionate producers and, as suggested for monensin (Van Nevel and Demeyer, 1977; Chen and Wolin, 1979), avoparcin most probably lowers methane production by reducing the supply of hydrogen available to the methanogens. Partial inhibition of methane by avoparcin has been observed in *in vitro* studies (R. C. Macgregor and D. G. Armstrong, unpublished observations).

Reference has already been made to the effectiveness of thiopeptin in preventing lactic acidosis in cattle and sheep. It has been shown *in vitro* (Muir and Barrets, 1979) that the antibiotic is very effective against *Streptococcus bovis*, a Gram-positive coccus and major lactate-forming bacterium of the rumen. The organism proliferates on available carbohydrate producing lactic acid; the resulting decline in rumen pH favours further proliferation of lactobacilli species. Dennis, Nagaraja and Bartley (1981), also using *in vitro* studies, found that the two ionophores monensin and lasalocid inhibited major lactate-producing organisms, with the exception of the *Selenomonas* spp., and in *in vivo* studies Nagaraja *et al.* (1981) have shown that both antibiotics are effective in preventing induced lactic acidosis in cattle.

MODE OF ACTION WITHIN THE SMALL INTESTINE

Gut microorganisms can interfere with the relationship between amounts of nutrients entering the small intestine and nutrient supply to the host animal (Vissek, 1978; Coates, 1980; O'Connor, 1980). Thus, with lipids it has been shown (see Coates, 1980, for references) that gut microorganisms can hydrogenate unsaturated fatty acids and thus may affect the uptake of the essential fatty acids; furthermore, saturated fatty acids are absorbed less effectively than unsaturated fatty acids. Bile acids and sterols are extensively modified by microbial action and some of the metabolites formed, such as lithocholic acid, can be harmful.

One of the characteristic features of ruminants is the very extensive recycling of nitrogen, primarily as urea, into the gastro-intestinal tract. What is not so widely realized is that, on conventional high-forage diets, the major part of this recycled nitrogen enters the tract post-rationally (Armstrong, 1980). Vissek (1978) has drawn attention to the possibility that high levels of ammonia-nitrogen, released by the action of bacterial ureases acting on urea, may be harmful to cell metabolism at the site of absorption. For evidence relating to the effect of the gut microflora on supply of vitamins and trace minerals, the reader should consult Vissek (1978) and O'Connor (1980).

Gut microflora also have an effect on the structure and function of the gut wall. One of the characteristic features of animals fed low levels of antibiotic is a thinning of the wall of the small intestine. These changes are seen in germ-free chicks, although are less marked (Coates, 1980). The villus is generally more slender, with a narrower lamina propria and more numerous microvilli. Of likely equal significance is the evidence that, in the germ-free chick, rate of renewal of the epithelial cells is slower. The importance of metabolism in the small intestine including epithelial cell turnover is well recognized (Armstrong, 1982; Lindsay and Armstrong, 1982) and it may well be that some part of the increased efficiency of energy utilization noted when low levels of an antibiotic are included in the feed is associated with a reduced cost of metabolism in the epithelium of the small intestine.

With reference to the process of absorption, Coates (1980), in concluding on the basis of work largely based on *in vitro* preparations, that absorption of some nutrients might be impaired by the presence of microorganisms, has stressed the need for studies on intact animals. In this connection, studies in sheep equipped with intestinal re-entrant cannulae (Macgregor and Armstrong, 1982) have shown that when the feed contained some 45 mg avoparcin/kg complete feed, apparent absorption of amino acid nitrogen entering the small intestine (expressed as a proportion of the amount entering the small intestine) rose from 0.597 to 0.687 (significant, $P < 0.05$).

Other mechanisms proposed as possibly contributing to the growth-promoting effect of antibiotics (Vissek, 1978) include (1) the possibility that microorganisms responsible for mild but unrecognized infections are suppressed, (2) microbial production of growth-depression toxins is reduced, (3) the antibiotic reduces microbial destruction of essential nutrients, and (4) the antibiotic encourages the synthesis of vitamins or growth-promoting factors which subsequently become available to the host animal. Virtually nothing is known concerning the significance of these mechanisms in the ruminant

animal. Equally, nothing is known concerning the effect that the presence of antibiotic in the caecum and colon of the ruminant may have.

Concluding remarks

It is clear that there is a role for non-clinical antibiotics as feed additives for growth promotion and/or improvement in feed conversion efficiency in growing/fattening ruminants. Important considerations that must be taken into account, relating to the use of such compounds, are the need to ensure the very minimal contamination of the meat produced and the need to eliminate from use any such antibiotics that have the property to induce resistance by particular bacterial species to the antibiotic or other antibiotics. Both of these important topics have been considered in Chapters 17 and 39.

Future research may yet show that antibiotics have a significant role to play in milk production. It is clear, however, that much yet remains to be understood concerning their mode of action before the full potential of antibiotics as feed additives for ruminant livestock can be realized.

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THE SELECTIVE CAPACITY OF PIG FEED ADDITIVES AND GROWTH PROMOTANTS FOR COLIFORM RESISTANCE

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Antibiotics have been used for several decades in human and veterinary fields in the treatment of bacterial infections. Studies of the medical use of antibiotics have concluded that there is a component of over-use, extending even to abuse. In the veterinary/agricultural areas, substantial amounts of antibiotics are used for non-therapeutic purposes, mainly as growth promotants in the intensive rearing of livestock. This practice has provoked numerous reports, some of which have considered the effect of antibiotic residues in tissues and on the emergence of resistance in the flora of the meat consumer. Other reports have discussed the possibility of environmental contamination by run-off from effluent discharge from piggeries and feed lots. The question of a salmonella carrier state in animals fed sub-therapeutic concentrations of antibiotics remains unresolved. The Swann Committee Report provoked a large number of investigations in this general field, and reports of the effectiveness of its recommendations will continue for many years. The Swann Committee made their decisions lacking quantitative data concerning whether an antibiotic selects for resistance. In the present author's opinion, it is more important to know this than whether an antibiotic is used in human medicine.

In this chapter, some research on the ability of a growth promotant to select for antibiotic resistance in the intestinal flora of pigs will be described. The aim has been to assess whether all growth promotants are totally safe, or equally bad, or whether some growth promotants are selective while others are safe. This is an area of research for which there is little comparable and extensive data. Few people have been prepared to carry out the amount of work required to measure these selective pressures, while those who have attempted it have found that herds available to them already have such high incidences of antibiotic resistance that the growth promotants produced results which were difficult to interpret reliably.

The present work has been conducted with Large White litters from the State Government Pig Research Station at Medina in Western Australia. The research station is managed along normal commercial lines in most of their pig husbandry procedures and is also used as a demonstration piggery for local pig farmers. For many years, one of the main projects at the station was a nutritional study using lupins as a feed base for pigs. In order to obtain data for that programme, unaffected by outside influences, growth promo-

tants were not used in rations for growing piglets. It was a very fortunate circumstance to find a piggery in which the level of antibiotic resistance in the herd was 100-fold, or more, lower than that frequently encountered elsewhere. It has been, therefore, an extremely sensitive indicator population. It could be argued that data on this herd is atypical, thereby invalidating any results. However, this is not the case, as although the incidence of resistant bacteria is low, those resistant bacteria present contain the same basic resistance principles as resistant bacteria in other piggeries. Furthermore, the incidence of resistance was found to respond in the expected way to tetracycline, an agent known to be highly selective for antibiotic resistance.

Experimental methods used to quantify selective capacity of growth promotants

The protocol has been to add a growth promotant to the feed, at the concentration which the manufacturers recommend as the 'nutritional' level, although some were used at the therapeutic concentration (*Table 28.1*). Litters for the study came from a section of the Medina herd every 3 months. They were weaned between 4 and 5 weeks of age and fed a weaner 'creep' feed *ad lib*. They were then transferred entirely to supplemented ration at week eight. Treated groups had supplemented feed for 6 weeks and were then supplied with the standard ration until market weight. Control groups were included in each batch, together with two treatment groups. Eight to twelve animals were housed in pens approximately 1.8 × 3.6 m with slatted flooring 1.8 × 1.2 m at the end furthest from the feed. The pens were separated from one another by 1.2 m wide corridors or empty pens and each pen was served by

Table 28.1 CONCENTRATION OF GROWTH PROMOTANTS USED IN BASIC FEED RATIONS

<i>Growth promotant</i>	<i>Nutritional concentration</i>	<i>Therapeutic concentration</i>
Avotan	20	
Bacitracin	20	
Bayernox	100	
Dimetridazole		200
Dynamutilin	20	
Flavomycin	5	
Hygromycin		13
Lincomycin	22	
Lincospectin		Lincomycin + spectinomycin 22 + 22
Mecadox	50	
Neftin	10	
Neomycin		100
Neo-terramycin		Neomycin + terramycin 100 + 200
Nitrovin (Payzone)	10	
Oleandomycin	5	
Progen	100	
Roxalin	120	
Stafac	20	
Sulphadimidine	100	
Terramycin	10	
Tylan	20	100
Tylan + sulphadimidine		Tylan + sulphidimidine 100 + 100

different effluent pits. The final measure to prevent cross-infection between pens involved a bath of disinfectant beside each pen. Two staff who attended these animals were careful not to transmit faeces between pens.

Fresh faecal samples were collected weekly from a pair of identified animals in each group. Sampling began at 2–4 weeks of age and was continued for as long as possible after the supplemented ration was withdrawn. This measured how long resistant bacteria could persist in the intestine once selective pressures were removed.

Within 2 h of collection, weighed amounts of each faecal sample were diluted in sterile buffered saline and aliquots plated on MacConkey agar. The composition of the media allowed antibiotic-resistant and sensitive coliforms and related organisms to grow.

The media also included, separately, the antibiotics ampicillin (A), tetracycline (T), chloramphenicol (C), kanamycin (K), streptomycin (S) and spectinomycin (P). Sulphafurazole (F) was incorporated in DST agar and the ions arsenate and mercuric (M) were incorporated in CLED medium. The concentrations used were sufficient to prevent the growth of sensitive bacteria, while allowing resistant strains to grow. A 20 µl Oxford sampler was used to dispense an aliquot of the dilution series to each of the selective media in a Miles and Misra operation. Dilutions to 10^{-5} were performed so that coliform concentrations ranging from 5×10^2 to 1×10^8 per gram faeces could be accurately determined. After 16–18 h incubation at 37°C, lactose-fermenting colonies were counted and the number of bacteria calculated. The count of colonies growing on each medium could be compared to the count on media without antibiotics. In order to measure whether colonies growing on MacConkey agar containing tetracycline were resistant to tetracycline only or to other antibiotics, 12 colonies were then tested for their ability to grow on the range of antibiotic-supplemented media. A similar number of colonies were examined from each of the selective media.

Results

Examples of 1 week's samples are presented in *Tables 28.2* and *28.3*. The number of coliforms per gram of faeces is shown under control and resistant colonies growing on each selective medium is presented in that row as a percentage of the control total. The incidence of the major resistance patterns, including sensitivity to all nine antimicrobial agents screened, is presented in columns for each selective medium. The final column is an average percentage for that resistance pattern, as determined from each selective medium. It can be seen that, in some faecal samples, resistant bacteria were present in very low numbers while other samples contained a population of bacteria all of which were resistant to several antibiotics. The data presented in *Table 28.2* was taken from the second week of a survey when neomycin and neomycin-terramycin mixtures were included in the supplemented rations. Lines 1 and 2 indicate the extent of antibiotic resistance in the untreated piglets. It was not difficult or rare to recover resistant organisms from these animals. However, most were resistant to a limited number of agents. Lines 3 and 4 illustrate the effects of neomycin plus terramycin. No sensitive organisms were detected

Table 28.2 INCIDENCE OF ANTIBIOTIC RESISTANCE AND PATTERNS IN PIGS ON UNSUPPLEMENTED RATION, NEOMYCIN - TERRAMYCIN RATION AND NEOMYCIN ALONE RATION

Resistance pattern	Control	A ^a	T	C	K	P	S	F	M	Averages
<i>Control</i>										
Fig 1										
TSF	5 × 10 ^{3b}	ND < 10 ^c	30	ND < 10	ND < 10	20	60	50	ND < 10	35
TPFM	25		20			5	60	33		4
T	8		5					0		6
Sensitive	50		5							50
Fig 2										
SF	5 × 10 ⁴	ND < 1	ND < 1	ND < 1	ND < 1	ND < 1	1	1	ND < 1	0.5
Sensitive	100						0.5	0.5		100
<i>Neo-terramycin</i>										
Fig 3										
AK	5 × 10 ⁵	80	10	0.6	80	10	60	10	4	58
AKS	60	67			48					15
TKPSFM	8	13			20		20			10
TKSF	16		6		7	10	20	8	4	9
ATCKSFM	16		1		7		20	1		1
Sensitive			1	0.6				1		0
Fig 4										
AKS	5 × 10 ⁴	80	80	6	80	1	100	80	10	50
ATCKSFM	33	73			40		60			12
TKSF	25	7	20	6	20		8	25	2	30
TSEF	25		47		0		25	33	6	10
TKPSFM	16		13				8	42	2	3
Sensitive			0		13	1	0	0		0
<i>Neomycin</i>										
Fig 5										
KSF	5 × 10 ⁵	40	20	ND < 0.1	80	0.6	80	80	ND < 0.1	65
ATKSF	66				60		53	80		11
AKS	8	33	13		0		13			7
K	8	7			0		13			7
Sensitive	8				7					8
Fig 6										
KSF	1 × 10 ⁶	10	0.5	ND < 0.01	100	1	100	100	ND < 0.01	87
ATKSF	84				92		75	100		3
AKS	8	3	0.5		8		0	0		10
K	8	7			0		25			4
Sensitive	0				0					0

^a Figures represent the percentage incidence of resistance or resistant pattern (A, ampicillin; T, tetracycline; C, chloramphenicol; K, kanamycin; P, spectinomycin; S, streptomycin; F, sulphafurazole; M, mercuric).
^b Represents the number of organisms recovered per gram of faeces.
^c ND = not detected.

Table 28.3 INCIDENCE OF ANTIBIOTIC RESISTANCE AND PATTERNS IN PIGS ON UNSUPPLEMENTED RATION, LINCOMYCIN AND LINCOSPECTIN RATIONS

	Resistance pattern	Control	A ^a	T	C	K	P	S	F	M	Averages
Control Fig 7	KPF	5 × 10 ^{4b}	1	2	1	50	50	1	50	ND < 0.4 ^c	47
	ATCKPSFM	42	0.5	2	1	50	50	1	46		1
	Sensitive	58				0			4		58
Pig 8	T	1 × 10 ⁴	5	40	ND < 5	ND < 5	30	10	50	ND < 5	24
	TPFM	17		30			20		20		17
	PFM	17		10			10		9		9
	SF	8						4	17		7
	Sensitive	50									50
Lincomycin Fig 9	T	2 × 10 ⁶	0.05	15	ND < 0.01	ND < 0.01	1	0.1	1	0.5	12
	PFM	8		15			1		0.5		1
	Sensitive	92									92
Pig 10	KPF	3 × 10 ⁶	1.7	5	0.66	66	66	1.7	66	3	66
	PFM	75				66	66		55	1	4
	Sensitive	25					0		11		25
Lincospectin Fig 11	PFM	1 × 10 ⁷	20	20	20	25	100	40	100	100	65
	ATCKPSFM	84		15	20	25	66	40	58	50	26
	TPFM	16	20	5			16		42	50	5
	Sensitive	0							0	0	0
Pig 12	T	8 × 10 ⁷	0.25	50	0.25	0.18	100	2.5	100	100	35
	TPFM	58		46			16		25	33	42
	PFM	25					58	0	75	8	13
	PSFM	16					0	0	0	50	< 1
	ATCKPSFM	0	0.25	0	0.25	0.18	0	0	0	0	0
	Sensitive	0									

^a Figures represent the percentage incidence of resistance or resistant pattern (A, ampicillin; T, tetracycline; C, chloramphenicol; K, kanamycin; P, spectinomycin; S, streptomycin; F, sulphafurazole; M, mercuric).

^b Represents the number of organisms recovered per gram of faeces.

^c ND = not detected.

and resistance to ampicillin (80%), tetracycline (80% and 10%), kanamycin (80%), streptomycin (100% and 60%) and sulpha drugs (80% and 10%) was quite common. Major patterns of resistance included AK (~60), AKS (~15) and TKPSFM (~10) in one piglet. The other piglet in the same group had only slightly different resistance patterns (AKS ~50, TKSF ~30, ATCKSFM ~12). The final group of animals were treated with neomycin alone. The major differences to the previous group are the lower frequencies of tetracycline resistance detected and the absence of any chloramphenicol or mercury resistance. In both animals of this treatment group, pattern KSF was dominant. Resistance to kanamycin, an aminoglycoside very closely related to neomycin, was present in almost every isolate from the two treated groups.

A second example (*Table 28.3*) has been taken from animals in their second week of supplemented rations containing lincomycin or lincospectin. Sensitive organisms made up the majority of the intestinal coliform flora from both control and lincomycin-treated animals. Lincospectin-treated animals contained no detectable sensitive bacteria and the resistance pattern (PFM) was universal.

All trials extended over a period of approximately 20 weeks and tables of this nature have been derived for each week. Summaries of 8 weeks' data from four different trials are presented in *Tables 28.4-28.7*. In order that a quantitative figure could be used to define undesirable growth promotants, it was arbitrarily decided that if in 5 of the 6 weeks no sensitive bacteria was isolated, and if the sum of major resistance pattern averages exceeded 75%, then the growth promotant had a high selective index and was undesirable for use. Sulphadimidine, lincospectin, neomycin and neomycin + terramycin exceeded the two measures on all occasions (*Tables 28.4, 28.5 and 28.7*). The growth promotants found to have a high selective index in addition to the above were terramycin alone, tylan + sulphadimidine and progen. Growth promotants found to exert little or no selective pressure on the resistance of intestinal coliform flora include tylan, at both concentrations, stafac, flavomycin, mecadox, bayernox, dynamutilin, nitrovin, hygromycin, oleandomycin, avotan and bacitracin. Results with neftin, lincomycin, roxalin and dimetridazole were intermediate and require repeating to confirm their selective index (*Table 28.8*).

General conclusions

There are several conclusions which can be made in relation to this data. Mixtures of growth promotants are generally undesirable, while rotation of growth promotants, although not tested, may not be. The selective index of a growth promotant is unrelated to its use in human therapy and data derived from pigs may not apply to chickens, calves or man. Most agents banned by the Swann Committee from use for growth promotion because of their use in human medicine have a high selective index in pigs and are undesirable.

Table 28.4 EIGHT-WEEK PERCENTAGE INCIDENCE OF RESISTANCE PATTERNS IN TRIAL WITH LINCOMAX AND LINCOSPECTIN

	-2	-1	1	2	3	Weeks 4	5	6	+1	+2
<i>Control</i>										
T ^a					+	+				
PFM		1	+			3		45		
KPF	3	+	5	47	+					
ATCKSFM		3	2	+		7				
ACSF			+	+		3				
Sensitive	92	100	92	58	100	75		33		
T	6	+	+	24	11	+				
PFM	+	+	+	9	5					
SF	7	84	10	7	7	30				
TPFM	5	+	+	17	5			+		
TSF	2		+	+	+	+				
Sensitive	66	16	50	50	66	60		100		
<i>Lincomix</i>										
T	7		5	12	9		+	8	8	15
PFM	8	14	3	+	7	8		+	+	11
TPFM	3		4	+	5	2		+	+	9
SF	3	41	11	+	8	24	8	30	+	10
TSF		+	+	+	8		+	+	6	
Sensitive	84	58	75	92	66	66	92	40	17	50
T	6	16	33	+	2	+	30	9	6	5
PFM	+		2	4	4	3	4		26	5
TPFM	4	4	+		4	+	+	+	+	+
SF	+	+	11	+	4	5	19	9	3	5
TSF	+	+	+		+	+	+		4	+
Sensitive	84	42	42	25	58	92	8	33	58	66
<i>Lincospectin</i>										
PFM				65		22	44	31	63	
TPFM				5			14	14	5	
ATCKPSFM				26	8	75	18	14	4	
PSFM					42			10		
Sensitive			100	0	0	0	0	0	8	92
PFM				42	22	42	27	36	4	6
TPFM				35	16	27	6	15	4	7
ATCKPSFM				+		+		+		2
PSFM				17		2		4		
Sensitive			66	0	0	0	33	0	8	75

^a For key to antibiotics, see the first footnote to Table 28.3.

+ represents the percentage resistance pattern below 1% to the limit of detectability.

Table 28.5 EIGHT-WEEK PERCENTAGE INCIDENCE OF RESISTANCE PATTERNS IN TRIAL WITH NEOMYCIN ALONE AND NEOMYCIN-TERRAMYCIN

	-2	-1	1	2	Weeks		3	4	5	6	+1	+2
<i>Control</i>												
T ^a				6								
SF			20		6	22						+
TPFM				4	4	10						+
TSF				35		+						
ATS			15									+
Sensitive		100	83	50	92	66	100					100
T			10			7						3
SF			6	+	2	30						21
TPFM						5						4
TSF		1			4	+						2
ATS		8			4							
Sensitive		92	83	100	92	75	66					58
<i>Neo-terra</i>												
AKS		5	3	72						+	+	
TKSF				9	88	40	84	88			+	2
TKPSFM				10	6		10	4			+	
ATCKSFM				1			5	2			+	
Sensitive		92	83	0	0	0	0	0	0	0	100	33
AKS			32	50							+	2
TKSF			13	30	79	64	50	77			46	45
TKPSFM				5	10		20	14			14	11
ATCKSFM			16	12		4		4			2	+
Sensitive		92	0	0	0	0	0	0	0	0	8	17
<i>Neomycin</i>												
AK		65										
TKSF				15		11	45	1			44	2
TKPSFM					72	18	9	7			12	
KSF				65	23	31	26				11	
AKS						44	23	70			2	
Sensitive		25		8	0	0	0	0	0	0	17	43
ATS		63										
TKSF					30	8	6	26			20	24
TKPSFM					4	9	8	10			5	10
KSF				89	44	57	21	30			23	8
AKS				10	21	18	63	25			14	+
Sensitive		50		0	0	0	0	0	0	0	0	42

^a For key to antibiotics, see the first footnote to *Table 28.3*.
+ represents the percentage resistance pattern below 1% to the limit of detectability.

Table 28.6 EIGHT-WEEK PERCENTAGE INCIDENCE OF RESISTANCE PATTERNS IN TRIAL WITH DIMETRIDAZOLE AND DYNAMUTILIN

	<i>Weeks</i>									
	-2	-1	1	2	3	4	5	6	+1	+2
<i>Control</i>										
T ^a				+		22	5	7	17	10
SF		+	2		+			9		
AK		+	+		9					
PFM			+	+		3	4	32	16	26
TPFM						6	20	5	12	6
Sensitive		100	100	100	83	58	75	42	67	33
T		4	+			21	2	4	10	11
SF	8		14	50						
AK	85	+	45	33						
PFM		+	+			3				12
TPFM						2	25	+	5	3
Sensitive	17	92	33	17		42	67	92	58	75
<i>Dimetridazole</i>										
SF	+	51	+			8				7
TSF				65	59	29	95	+	+	3
AK		33	9							
PFM	+						+		+	
TPFM									+	7
Sensitive	100	17	83	33	41	67	0	100	92	58
SF		2	2	28		2				
TSF			+	4		2	81	100	+	2
AK		5	4	+						
PFM									4	7
TPFM									2	4
Sensitive		92	92	67	100	100	0	0	92	58
<i>Dynamutilin</i>										
T				+	+				5	2
SF	+			+	+					4
PFM			3	+	+	+	3	2	30	27
TPFM										6
TSFWM				+		2	3	+	3	
Sensitive	100	100	97	100	100	92	92	100	25	42
T			+	+					+	8
SF			+		+		+			
PFM				+	+	3	4	8	35	36
TPFM				+					3	4
TSFWM						+	5			
Sensitive		100	100	100	92	92	83	92	25	42

^a For key to antibiotics, see the first footnote to Table 28.3 (W, trimethoprim).

+ represents the percentage resistance pattern below 1% to the limit of detectability.

Table 28.7 EIGHT-WEEK PERCENTAGE INCIDENCE OF RESISTANCE PATTERNS IN TRIAL WITH TYLAN AND SULPHADIMIDINE

	-2	-1	1	2	Weeks		3	4	5	6	+1	+2
Control												
ACS ^a			4	63	+	+	+		21		14	
AKS	24				+	+			3		16	
TSF							2	+				
SF		75						+				
ATCS		16										
Sensitive	0	0	0	33	100	84	100		66		75	100
ACS		4		+	53	72	4		30		+	
AKS	25	11		+	12	+	+					
TSF					12							
SF										9		
ATCKSF	3		2			+	3		3			
Sensitive	17	0	17	100	0	0	84		25		25	100
Tylan												
ACS	+	43		+	+	3	+		11		+	18
AKS							10					
TSF	+				+	45	+		11		5	13
PF		18			+	5			2			
ATCKSF	+	11							3			
Sensitive	100	17	92	0	100	42	92		55		67	50
ACS		+	3		+	+	+					6
AKS												
TSF				+	+	+	+	+	+		+	5
SF				+			92	+				
ATCKSF	+											+
Sensitive	92	100	50	100	100	58	0	100		100		92
Sulphadimidine												
F			100								2	
SF	50			100					80			
TSF	+	+		3	75		14		5		+	+
TPF						27						
ATCKSF	4		+								2	+
Sensitive	0	100	0	0	0	0	100		33		100	100
F			92	100				100	85			95
SF	59					15					65	
TSF		6				33			15		4	
TPF		+										
ATCKSF		6	3			13			5			
Sensitive	0	58	0	0		50	0	0		0	0	0

^a For key to antibiotics, see the first footnote to *Table 28.3*.
+ represents the percentage resistance pattern below 1% to the limit of detectability.

Table 28.8 SELECTIVE INDEX

<i>High</i>	<i>Intermediate</i>	<i>Low</i>	
Neomycin	Dimetridazole	Avotan	Hygromix
Spectinomycin	Lincomycin	Bacitracin	Oleandomycin
Sulphonamides	Neftin	Carbadox and relatives	Payzone
Progen	Roxalin	Dynamutilin	Stafac
Tetracyclines	Roxazole	Flavomycin	Tylan

NUTRITIONAL ASPECT OF VIRGINIAMYCIN IN FEEDS

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Microorganisms are an integral part of the gastrointestinal (GI) tract of livestock. Ruminating livestock species have complex forestomach microflora that perform very essential nutritional functions for the host (Clarke, 1977). Major functions of such microflora include cellulose hydrolysis, conversion of non-protein nitrogen to essential amino acids and production of B-vitamins (Hungate, 1966). Rumen microbes also have detrimental effects on the host and have less than optimum effect on digestion of certain food components. Detrimental effects include lactic acidosis. Inefficiencies include loss of energy as methane gas, imbalance of rumen energy end-products, and degradation of essential amino acids. Scientists have attempted to manipulate the rumen ecosystem to improve the efficiency of rumen fermentation while maintaining the essential nutritional functions of the rumen microflora (Chalupa, 1980).

Non-ruminants also have extensive microbial populations that are unique to various segments of the GI tract. As in ruminants, microflora have beneficial and detrimental effects on the host (Coates, 1976). Virginiamycin has been applied as a manipulator of the GI microflora, with resulting improvement in growth and feed utilization efficiency of non-ruminating farm animals.

GI microflora—swine and poultry

Much has been learned in recent years of the microbial populations of the GI tract of swine and poultry (*Figures 29.1 and 29.2*). Fuller (1982) has summarized the data concerning the aerobic flora of the chicken and pig. Principal aerobic populations include *Lactobacillus* and *Streptococcus* species. Anaerobic populations are more poorly described and, in general, they populate the lower bowel rather than the crop-stomach and small intestine regions of the gut (Robinson, Allison and Bucklin, 1981). If one examines the GI tract in segments with respect to microbial populations, some generalizations can be derived:

- (1) Aerobic, Gram-positive organisms are the predominant species found in the crop-stomach and small intestine portion of the GI tract. Under

stressful conditions *Escherichia coli* can predominate, but this is an abnormal case (McAllister, Kurtz and Short, 1979).

- (2) Anaerobic, Gram-negative organisms are the predominant species found in the caecum and large intestine.

GI microbial effects—swine and poultry

Organisms of the digestive tract of poultry produce a net detriment to the host (Coates, 1976). In terms of growth and feed utilization, axenic chickens

Table 29.1 EFFECT OF MICROBIAL STATUS ON BIRD WEIGHT AND ENERGY AND NITROGEN RETENTION EFFICIENCY

	<i>Axenic</i>	<i>Lactobacillus challenge</i>	<i>Mixed culture challenge</i>
Empty bodyweight	359	347	313
Energy retained (%) ^a	38.7	31.9	34.0
Nitrogen retained (%) ^a	52.2	45.3	48.8

Source: from Szylit and Charlet (1981).
^a Retention percentage was the proportion of dietary energy or nitrogen that was retained as body tissue.

were superior to monoxenic (lactobacilli from the crop) and holoxenic chickens (Szylit and Charlet, 1981). Table 29.1 lists the differences in terms of 27-day weight gain and efficiency of energy and nitrogen retention.

The superior nutritional efficiency of germ-free birds occurs, even though GI microflora are known to possess many desirable nutritional functions. Coates (1976) has listed such benefits to include bacterial amylase for starch

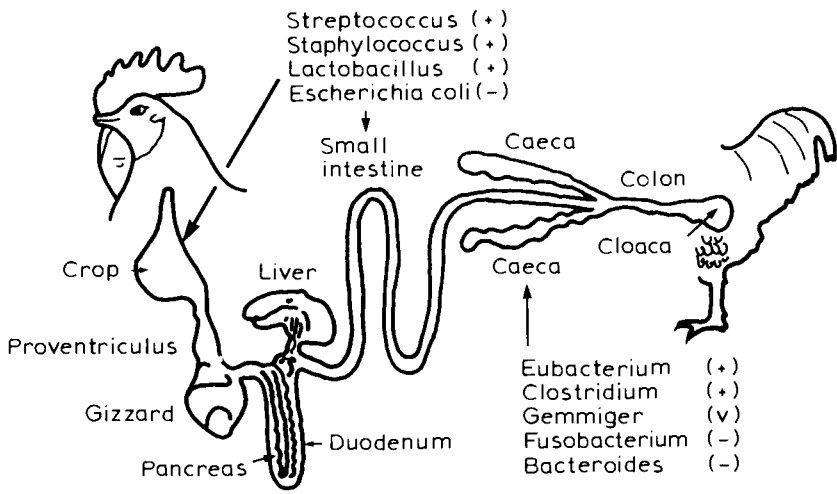


Figure 29.1 Microorganisms from the chicken's digestive tract. Gram stain characteristics are noted in parentheses

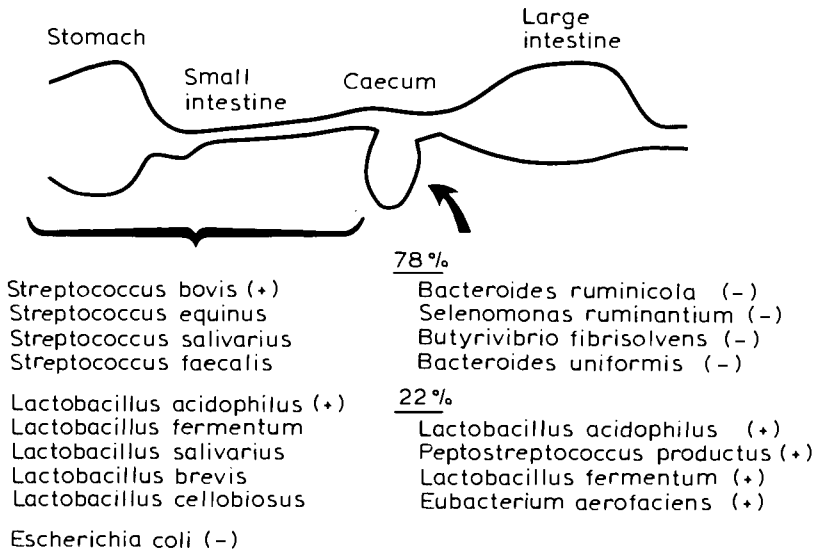


Figure 29.2 Microorganisms from the pig's digestive tract. Gram stain characteristics are noted in parentheses

digestion, recovery of endogenous nitrogen and synthesis of B-vitamins. Friend, Cunningham and Nicholson (1963) have suggested that 5-20% of the pig's energy requirement is derived from bacterial fermentation in the caecum and large intestine. When pigs were fed fibre-rich diets, such bacterial fermentation yielded energy from cellulose that would not be available with host enzyme systems. Thus, GI bacteria possess many beneficial attributes and yet their net result is negative.

Coates (1976) also lists potential detriments of GI microorganisms. The detriment specifically involves bacterial metabolites and reduced absorption of nutrients across the small intestinal epithelium. Intestinal stasis in dogs has resulted in bacterial overgrowth with subsequent poorer intestinal absorption efficiency (Hoenig, 1980). Saunders and Sillery (1982) have attributed fermentative diarrhoea to increased lactic acid levels in the small intestine of man. *Streptococcus faecalis* has been reported to cause reduced nutrient absorption in chicks (Coates, 1976). Gracey *et al.* (1975) report reduced small intestinal absorption in man when challenged with *Staphylococcus pyogenes*, *Streptococcus viridans*, *S. faecalis* and *Lactobacillus* spp. Gram-negative organisms such as *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp. also reduce intestinal absorption. Eyssen and DeSomer (1967) found *S. faecalis* to depress growth in gnotobiotic chicks, and this depression was corrected by virginiamycin feeding. Thus, detriment of GI microorganisms appear to involve bacterial metabolites and/or toxins that reduce nutrient assimilation from the small intestine. Vervaeke *et al.* (1979) have also considered the energy loss associated with conversion of glucose to lactic acid in the pig small intestine. Virginiamycin's suppression of glucose degradation was calculated to account for the majority of the feed utilization efficiency improvement noted with virginiamycin feeding.

GI microflora—manipulation

There are definite benefits and detriments associated with GI microflora. When considering reports of germ-free birds and their superior nutritional efficiency over conventional birds, one could conclude that the ideal condition would be a sterile gut. However, as described earlier, caecal microorganisms can provide beneficial functions that should be utilized for maximal energy efficiency, particularly with fibrous diets.

VIRGINIAMYCIN—A GI MICROFLORA MANIPULATOR

Investigations have been made of the virginiamycin effect on small intestinal microflora. During these investigations, more than 100 pigs were cannulated in either the ileum or caecum. Pigs were sampled over a 20–100 kg weight range. To assure that changes in microflora were related to the pigs' weight/age rather than diet, a constant diet formula of 16% crude protein, based on corn-soya bean meal ingredients, was fed throughout the experiment.

In general, the previously reported spectrum of GI microbial activity was found in this study. Small intestinal microorganisms fermented maltose to lactic acid, with a lesser portion of volatile fatty acids produced as well (Hedde *et al.*, 1982a). Caecal microflora fermented maltose and cellobiose; however, the primary products were volatile fatty acids rather than lactic acid (Figure 29.3).

There were weight/age related effects discovered that add to the literature on this topic. In the small intestine the microbial population changed with

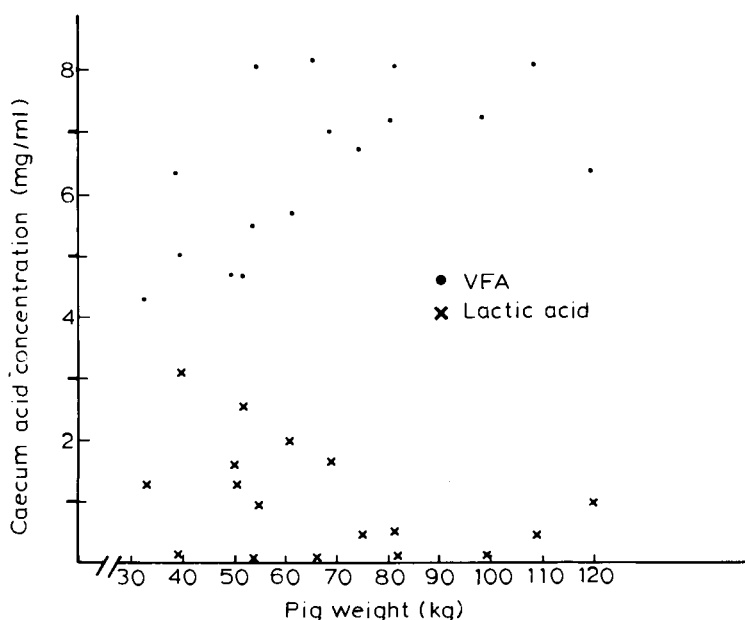


Figure 29.3 Concentration of volatile fatty acids (VFA) and L-lactic acid in caecal contents of growing-finishing weight pigs

weight/age such that lesser lactic acid production occurred with no changes in volatile fatty acid production. Thus, the proportion of lactic acid decreased from 80% to 50% of the total fermentation acids, as pigs grew from a 30 to a 100 kg weight.

It was further noted that, as the microbial population shifted, the propensity for the culture to remove lysine from the incubation media increased from nearly zero in the 30 kg pig to 0.05 (mg/ml)/h in 100 kg pigs (Figure 29.4). From this study it appears that small intestinal microorganisms compete for energy in the early part of swine production and compete for lysine as pigs

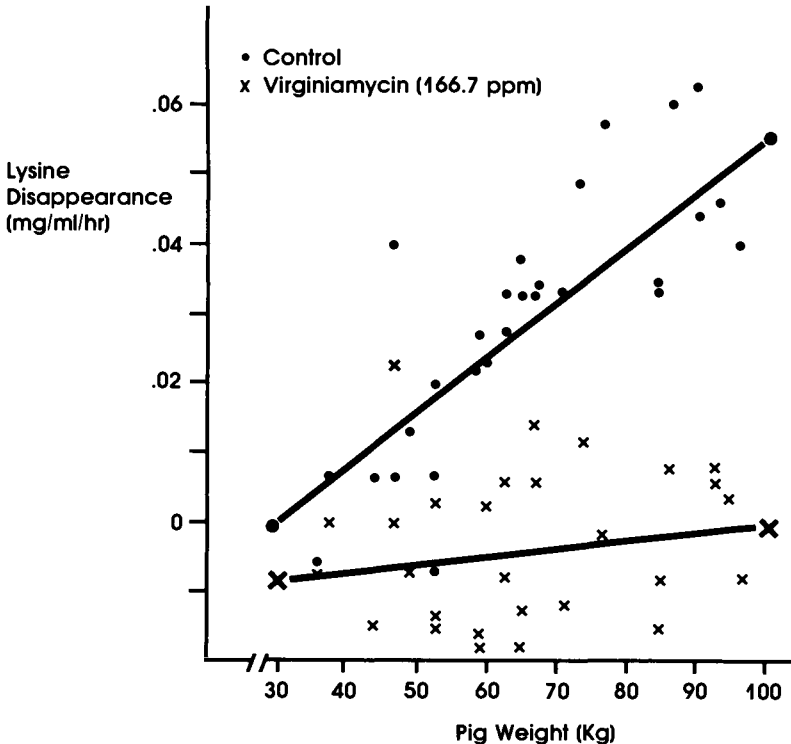


Figure 29.4 Lysine removal from ileal contents *in vitro*. Effect of inoculum source (pig weight) and virginiamycin

approach market weight. Lysine is the most limiting amino acid for swine growth in corn-soya bean diets, and any microbial competition could compromise the pigs' growth rate.

Virginiamycin reduces the nutrient competition deriving from small intestinal bacteria. In terms of energy, virginiamycin reduces the fermentation of glucose by small intestinal bacteria (Figure 29.6). Lysine destruction is also prevented by virginiamycin (Figure 29.4).

Virginiamycin has very desirable effects towards decreasing the lysine and energy competition brought by microbial populations in the pig small intestine. As noted earlier, metabolites such as lactic acid may have adverse effects on intestinal epithelium. Although not determined here, lysine degradation products, such as cadaverine, can also be toxic to the host. Thus, the strict

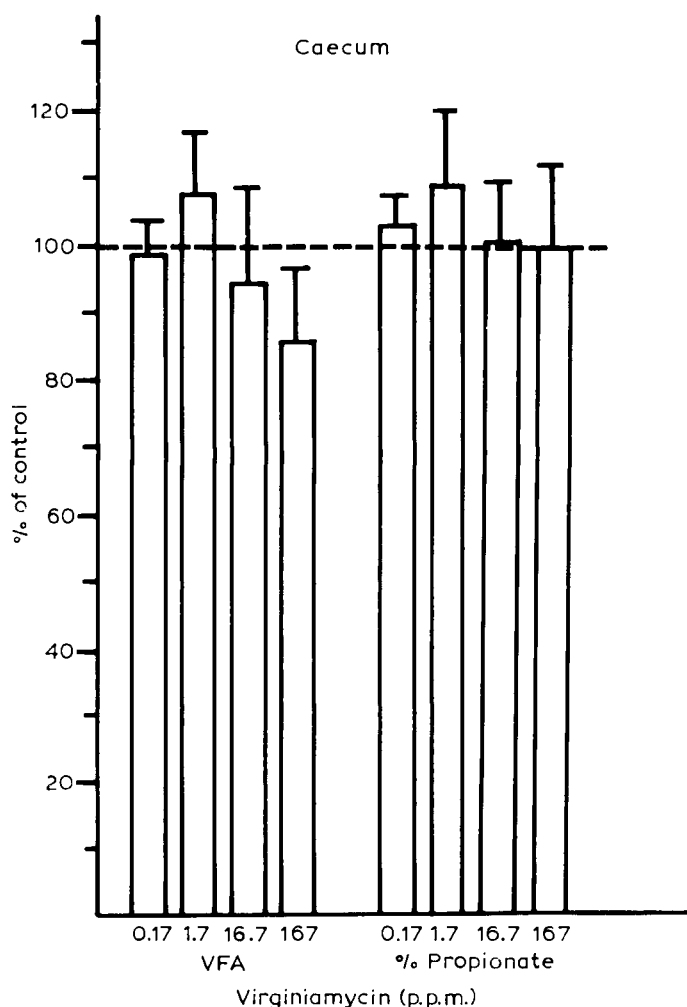


Figure 29.5 Virginiamycin effect on volatile fatty acids produced by caecal microorganisms *in vitro*. The proportions of volatile fatty acids produced are characterized by the propionate percentage

nutritional impact of virginiamycin in the small intestine may further benefit the host in terms of reduced GI toxins.

Caecal microbial population also change with weight/age (*Figure 29.3*). In the light pig, considerable lactic acid fermentation occurs in the caecum. In the 70–100 kg pig, caecal fermentation is primarily involved with volatile fatty acid producers. Species of bacteria that produce volatile fatty acids tend to be better cellulase producers than the lactic acid producers. Virginiamycin reduces the lactic acid aspect of caecal fermentation without restricting volatile fatty acid fermentation (*Figure 29.5*). Since volatile fatty acids can be absorbed across the caecum and large intestine of pigs (Argenzio and Southworth, 1975) and chicken (Gasaway, 1976), they represent a source of energy to the host that would be unavailable in the germ-free animal.

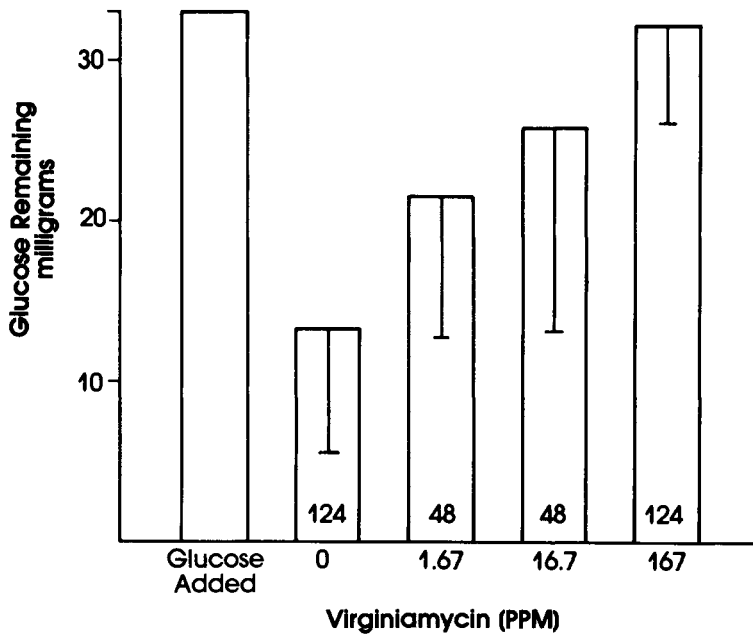


Figure 29.6 Virginiamycin effect on glucose fermentation by swine ileal contents *in vitro*. Numbers within bars refer to the number of incubations used in developing the dose mean and standard deviation

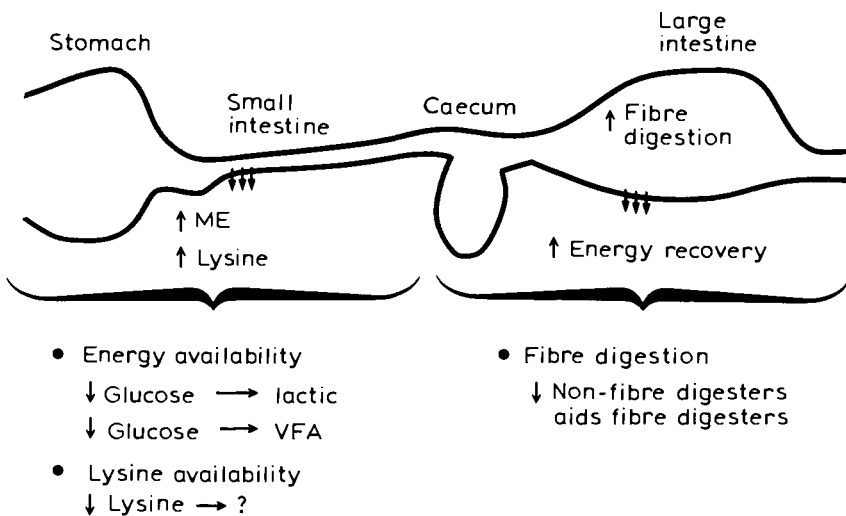


Figure 29.7 Virginiamycin effect on gastrointestinal microflora and potential nutritional benefit (ME, metabolizable energy)

The digestive tract is unique in that it provides environments such that very different microflora populate sites in the tract, of close proximity. It is remarkable that microflora in the terminal ileum are vastly different from microflora in the caecum, with just a few centimetres separating the tract regions. It is equally remarkable that an antibiotic such as virginiamycin can suppress the microflora of the ileum with essentially no detriment to the organisms of the caecum. *Figure 29.6* shows the ability of virginiamycin to prevent the fermentation of glucose by microorganisms. Production of acids during fermentation in the ileum is blocked by virginiamycin, while production of acids in the caecum is only slightly inhibited by virginiamycin (*Figure 29.5*). *Figure 29.7* summarizes the virginiamycin effect as projected from swine *in vitro* studies. The lessened fermentation of glucose and lysine by the small intestine would yield greater metabolizable energy and lysine for the host when virginiamycin is included in the diet. Also, the lessened lactate fermentation in the caecum would yield an environment more conducive to fibre digestion by caecal microorganisms.

GI manipulation—practical benefit

Virginiamycin has been reported to increase growth rate and improve feed utilization efficiency in veal calves, swine, poultry (Cocito, 1979) and, more recently, in ruminating cattle (Hedde *et al.*, 1982b). It has been noted, in pharmacokinetic studies, that virginiamycin is poorly absorbed from the GI tract (Cocito, 1979) and thus it is approved for feeding continuously through the marketing process. The poor GI absorption aspect of virginiamycin suggests that its benefit to animal productivity would not be related to respiratory infections or other infections that would be treated by systemic antibiotics. Thus, the manipulation of GI microflora by virginiamycin and resulting growth improvement seems quite probable.

Recently, virginiamycin has been extensively studied at 21 US and Canadian universities and industrial research sites. Of the 21 tests, a positive growth improvement was noted in 17 tests and 19 of 21 tests were positive for feed utilization efficiency. On average, a 4.0% improvement in daily gain and a 3.9% improvement in feed utilization efficiency were produced by virginiamycin.

In poultry, virginiamycin also enhances growth and feed utilization, and does so in the presence of coccidiostats and arsenicals (Hochstettler, 1981). Metabolizable energy level of the diet appeared to be a factor in the increment of virginiamycin response. That is, low-energy poultry rations (5% lower metabolizable energy), were more responsive to virginiamycin than high-energy rations. Such effects can assist the producer in applying lower energy feedstuffs to his poultry feeding programme.

Conclusions

Knowledge of the GI microflora is improving with new understanding of GI tract segmentation from a microbiological viewpoint as well as a digestive function viewpoint. Non-ruminant animals consume fewer feedstuffs requir-

ing microbial fermentation of cellulose during digestion than do ruminants. Nevertheless, digestion provides a very rich energy and nitrogen food supply for bacteria in the small intestine of swine and poultry. The economic needs of livestock production require that nutrients be efficiently used for growth by the host and that unnecessary bacterial competition in the GI tract should be minimized. Sterilization of the gut is not a reasonable alternative, even though germ-free animals are more efficient and have more rapid growth rates than animals with a GI microflora. Caecal and large intestinal microbes serve positive nutritional roles for the host, and gut sterilization would remove their benefit.

Manipulation of the GI microflora with virginiamycin is a more practical alternative. Virginiamycin reduces the negative energy and lysine aspects of small intestinal microorganisms without detriment to the positive attributes of caecal and colon organisms. The result is improved growth, egg production and feed utilization efficiency in swine, poultry, veal calves and beef cattle.

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SALMONELLA SHEDDING AND FEED ADDITIVES

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Concern over potential salmonella in the human food supply has led to the consideration of a variety of measures for its control. Within the past decade, the effect on salmonella of antimicrobial additives in animal feed has come under scrutiny. It has been reported in the medical literature that antibiotics having antimicrobial activity against salmonella *in vitro* were ineffective in treating human cases of enteric salmonellosis (MacDonald, Friday and McEacharn, 1954). Indeed, the observation was made by Aserkoff and Bennett (1969) that a therapeutic course of ampicillin or chloramphenicol seemed to prolong the disease.

Competitive exclusion and antibiotics

In the early 1970s, a group of investigators in Helsinki headed by Professor Nurmi examined the effect of antimicrobials on the levels of *Salmonella infantis* in young chicks. They had shown that enteric microflora from adult chickens, when given orally to newly hatched chicks, served to inhibit colonization by salmonella (Rantala and Nurmi, 1973; Nurmi and Rantala, 1973; Rantala and Nurmi, 1974; Rantala, 1974a). If the suspension of enteric microflora from adult chickens were incubated *in vitro* with certain antibiotics, the capacity of the mixture to inhibit salmonella was reduced. It was postulated that critical organisms were destroyed or inhibited by the antibiotic and were then unavailable to colonize young chicks and interfere with salmonella colonization. The use of adult microflora to render young chicks resistant to salmonella was quickly confirmed in other laboratories in Australia, Canada, England, the USA and Germany. This phenomenon came to be termed 'competitive exclusion' or sometimes more simply, the 'Nurmi concept'.

Rantala (1974b) reported that oxytetracycline, when mixed *in vitro* with competitive flora, interfered with the expected protective effect when organisms were inoculated orally into chicks. In the same study, she showed that nitrovin, a growth-promoting antimicrobial, had no such inhibitory effect. She extended these observations by showing that, *in vivo*, oxytetracycline tended to promote the establishment of salmonella in chicks, whereas birds

receiving nitrovin in the diet for 5 weeks showed a slightly reduced level of *S. infantis*. The group from Finland continued to examine the effect of antimicrobials and reported that zinc bacitracin in the poultry diet showed a lower incidence of salmonella than the control group (Nurmi and Rantala, 1974) and that furizolidone fed intermittently potentiated salmonella establishment, whereas continuous treatment with this antimicrobial had no such adverse effect. Furizolidone was shown to have no suppressive effect on salmonella if initiated in the diet after colonization by salmonella.

US studies of tetracyclines in animal feeds

It was during the period 1973–75 that the effect of tetracyclines on salmonella in poultry, swine and calves was examined in the USA. The Food and Drug Administration (FDA) required that the industry sponsors of feed tetracyclines should carry out studies to determine whether or not the additives promoted shedding of salmonella in meat animals. This work was recommended by the FDA Task Force, a group of experts from the government and the academic world. The Task Force was formed in 1970 in response to the concern over public health consequences of the use of antimicrobial feed additives. It was formed soon after the UK 'Swann Report' from the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine was issued. Although the Swann Report discussed antibiotic resistance in salmonella, it did not consider the possibility of salmonella potentiation by antimicrobials in feedstuffs. The FDA Task Force cited the clinical work of Aserkoff and Bennett (1969) and required that this question be addressed.

The four sponsors of tetracycline feed additives in the USA independently carried out 11 studies in swine, calves and chickens to determine whether or not their products increased salmonella shedding. In each case, a very high challenge level of *S. typhimurium* was used since the FDA also required that the work address the question of the transfer of R-plasmids from indigenous microflora to the challenge organism. It was necessary to use antibiotic-sensitive salmonella and to infect with a very high level to maximize the number of potential plasmid recipients. Only those results which deal with salmonella shedding in chickens are described here.

Evangelisti *et al.* (1975) challenged 5-day-old chicks with more than 10^{11} *S. typhimurium* per chick and followed the course of infection in birds receiving 200 g/ton* oxytetracycline, as well as those receiving a control diet. *Figure 30.1* summarizes the results and it may be seen that the medicated birds showed lower levels of the challenge organism. Similar work was reported by Jarolmen, Shirk and Langworth (1976), with chicks fed chlortetracycline at 200 g/ton showing no significant differences from controls. They also described a study in which the spread of salmonella from infected to non-infected chicks was measured. Chlortetracycline at 200 g/ton was shown to reduce the spread of *S. infantis*, *S. enteritidis* and *S. typhimurium* from 2 infected chicks to 50 non-infected. It should be noted that in some of the poultry tests reported to the FDA, the challenge salmonella picked up R-

* 1 ton = 1.016 05 tonnes.

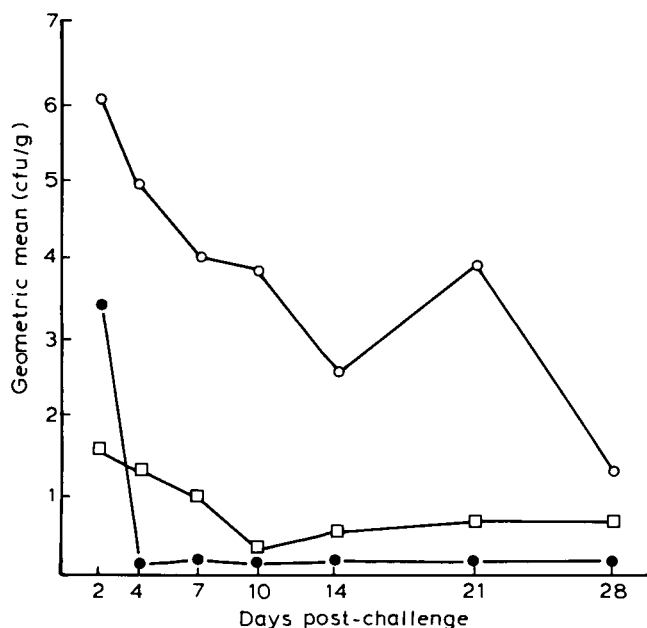


Figure 30.1 Concentration of *Salmonella typhimurium* in chick excrement following challenge: ○—○, control; ●—●, oxytetracycline and neomycin; □—□, oxytetracycline (after Evangelisti *et al.*, 1975)

plasmids from the indigenous microflora and, in one of the three studies, the tetracycline-treated group had a greater incidence of antibiotic resistance than controls. This was not true of the eight salmonella studies carried out in calves and pigs.

English studies

The studies described to this point have used a relatively small number of chicks and, in most cases, the level of salmonella in the excreta was measured in individual birds. A series of studies was carried out at the Houghton Poultry Research Station (Smith and Tucker, 1975a; 1975b; 1978; 1980) that was much more ambitious in the number of antimicrobials examined and the numbers of chicks utilized. The first of these papers described the consequences of using therapeutic antibiotics in poultry feeds at levels for most drugs of 100 p.p.m. and 500 p.p.m. (Smith and Tucker, 1975a). Trimethoprim was tested at 20 and 100 p.p.m. At the higher concentration, trimethoprim/sulphadiazine, neomycin, furazolidone and ampicillin reduced salmonella for the 9-day medication period, but levels equivalent to unmedicated controls were restored soon after the antimicrobials were withdrawn from the diet. Spectinomycin, chloramphenicol, oxytetracycline, streptomycin, neomycin and polymyxin were ineffective in reducing salmonella at both concentrations tested. In no case was salmonella increased where these dietary supplements were used.

A study reported at the same time by Smith and Tucker (1975b) examined the effect of permitted feed additives on salmonella shedding in poultry. With

the exception of sulphaquinoxaline, all of the tested additives have no significant anti-salmonella activity *in vitro* and were not expected to reduce salmonella, except possibly by indirect means. The purpose of the study was to determine whether or not the microflora of chicks was disturbed sufficiently to allow a more receptive environment for salmonella. Additives were tested at 100 p.p.m. and 10 p.p.m. in the diet. Conclusions drawn were that antimicrobials having no significant Gram-negative activity could increase salmonella shedding. Antimicrobials which could induce a large increase in salmonella were reported to be nitrovin, flavomycin and tylosin. Virginiamycin and zinc bacitracin showed either no effect or a slight effect.

A few years later, Smith and Tucker (1978; 1980) extended their work to include similar studies with avoparcin, lincomycin, dimetridazole, arsenilic acid, amprolium, monensin and nitro-hydroxyphenylarsonate. They used two basic models: in one, groups of 33 chicks were infected when 4 days old and the prevalence of salmonella shedding was monitored for several weeks; in the other, the researchers infected 5 chicks and placed them in contact with 28 non-infected chicks. The spread of the challenge organism to non-infected chicks was recorded. Both models were reported to show increased salmonella shedding in chicks receiving avoparcin or lincomycin in the diet. The additives reported to promote salmonella in Smith and Tucker's 1975 publications were retested in the spreading model and the potentiating influence of tylosin and nitrovin was confirmed.

In 1980, the phenomenon was reported with a variety of poultry diets, salmonella serotypes, broiler breeds, and in deep litter in a floor-pen trial. The previous work at Houghton had been carried out on wire screening in batteries. The decision to examine a wide variety of conditions in the last series of studies by Smith and Tucker made it necessary to test only three feed additives: one that had previously promoted salmonella (avoparcin); one that had only a slight or no effect (zinc bacitracin); and one that had been shown to reduce salmonella shedding (arsanilic acid). The studies confirmed the results of their previous investigations with these additives.

German studies

Recently, Matthes, Leuchtenberger and Lölig (1982) reported the results of battery tests in which relatively few chicks were challenged at 1 day old with low levels of *S. typhimurium*. They concluded that all three antibiotics tested extended the period of salmonella shedding when compared to controls. Additives were virginiamycin, avoparcin and tylosin. The same authors have also reported that certain organic acids used as poultry feed preservatives in Germany extended salmonella shedding (Matthes, Leuchtenberger and Lölig, 1981). In contrast, studies with virginiamycin were carried out by Abou-Youssef and DiCuollo (1982), who concluded that chicks fed this antibiotic did not show increased salmonella shedding when compared to a control diet.

Field surveys

Most of the studies described to this point have been carried out in batteries

with relatively low numbers of chicks raised under conditions which appeared to be significantly different from those encountered during the production of broilers. Several attempts were made to monitor naturally occurring salmonella in flocks in England which were somewhat closer to production conditions. In nearly all of these studies, there was an insufficient incidence of salmonella to allow conclusions. One exception was a study in turkeys in which *S. hadar* was encountered (Smith and Green, 1980). Unfortunately, all turkeys were treated early in the study with chloramphenicol for a respiratory infection. Nevertheless, this therapeutic incident did not reduce the prevalence of salmonella and no differences were seen between turkeys receiving avoparcin, virginiamycin or a control diet after 84 days.

Following our limited success in surveying poultry in England, studies were carried out in Germany and the precaution was taken to examine flocks in which salmonella had been monitored and was known to exist. In three surveys, a total of 2800 caecal samples were taken from 306 000 broilers raised in production. The first two surveys compared birds getting virginiamycin and avoparcin, and no differences were detected between these groups. Unfortunately, it was not possible to include a nil control. The third survey showed no differences in salmonella incidence in groups receiving avoparcin or a control diet. In all three production studies, a coccidiostat was used as part of the diet. Several serotypes were detected in each survey and the variation between replicates was very high. The results of these surveys for natural salmonella in Germany have not been published.

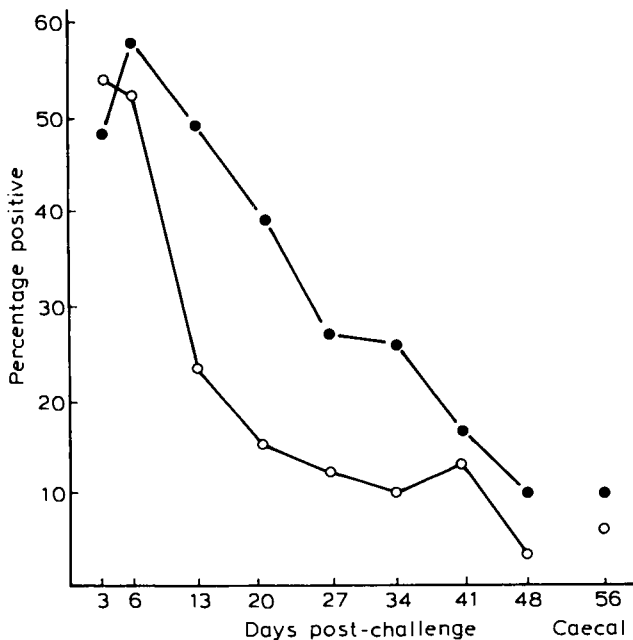


Figure 30.2 Percentage of chicks positive for *Salmonella typhimurium* after challenge with 10^6 cfu at 4 days of age: ○—○, control; ●—●, avoparcin, 10 p.p.m. (from Gustafson, Beck and Kobland, 1982, by courtesy of Paul Parey Verlag, Berlin)

US studies

In studies carried out in Princeton, models similar to those of Smith and Tucker were used, but efforts were made to bring conditions closer to those encountered in production. Floor-pen trials on deep litter were carried out and monensin was included in the diet because of its wide use as a coccidiostat. A moderate level of salmonella was used as challenge. A dose sufficient to infect 50% of the broilers was considered reasonably close to the maximum level encountered by most broilers. The number of chicks per group was quite large compared with most previously published studies. This was considered necessary, since experience had shown considerable variation between replicates, even when the salmonella dose was carefully controlled. A nalidixic acid-resistant strain of *S. typhimurium* was used in all studies (Gustafson, Beck and Kobland, 1982).

The first study (Figure 30.2) utilized 150 chicks per treatment group, 3 replicates per group. Salmonella for all ante-mortem sampling intervals was determined by cloacal swabs using appropriate selective medium containing nalidixic acid. Direct plating followed by liquid enrichment was utilized for all samples. It may be seen that, in this study, chicks in both groups rapidly became negative for salmonella, with the avoparcin group lagging behind the

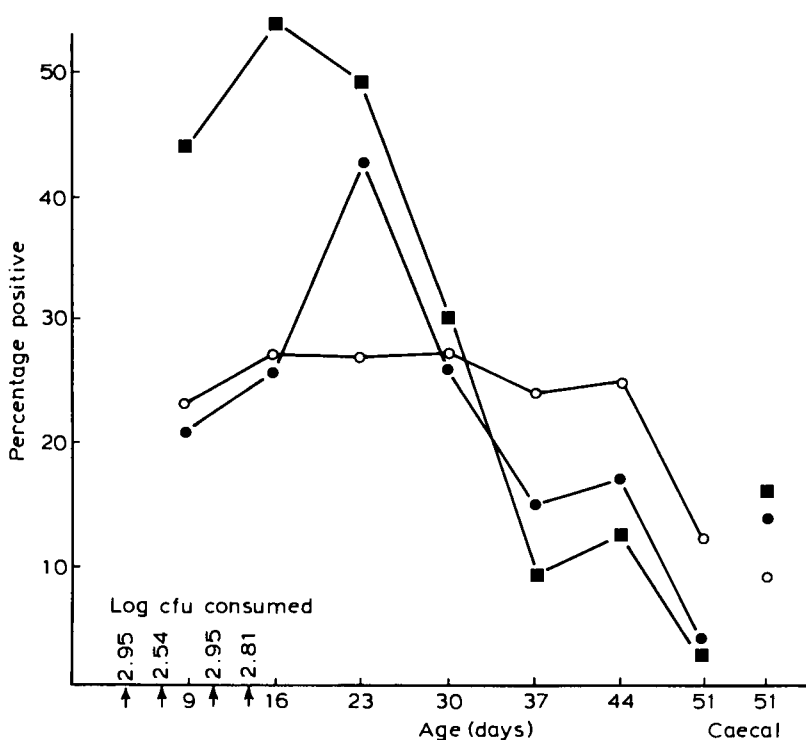


Figure 30.3 Percentage of chicks positive for *Salmonella typhimurium* following low-level challenge in drinking water at 4, 7, 11 and 14 days of age: ○—○, control; ●—●, avoparcin, 10 p.p.m.; ■—■, virginiamycin (from Gustafson, Beck and Kobland, 1982, by courtesy of Paul Parey Verlag, Berlin)

control group during the intermediate stages. As chicks approached slaughter age, no significant differences were seen in prevalence.

The next study (Figure 30.3) utilized the drinking-water as a means of salmonella challenge. During 7 h exposure periods at 4, 7, 11 and 14 days of age, each chick consumed approximately 3×10^3 viable salmonella. Both virginiamycin and avoparcin were included as test groups. Samples taken on days 9, 16 and 23 showed somewhat more salmonella in the virginiamycin group and on day 23 for the avoparcin group, but later samples from these groups showed reduced salmonella when compared to controls. It was concluded that there was no appreciable effect of these feed additives on the establishment of salmonella. A study was carried out in our laboratory in which the chicks were older when challenged. This test was modelled after one reported by Benazet and Cartier (1980) with nosisheptide. Avoparcin was added to the diet at 16 days of age and chicks were challenged at 21 days with approximately 10^6 organisms (Figure 30.4). A rather consistent shedding pattern was noted, with no effect of the feed additive.

The differences between production conditions and laboratory models had been a disturbing element in all of our deliberations for some time. It was clear that many variations in experimental conditions could influence the outcome. It is also apparent that resistance to salmonella colonization in-

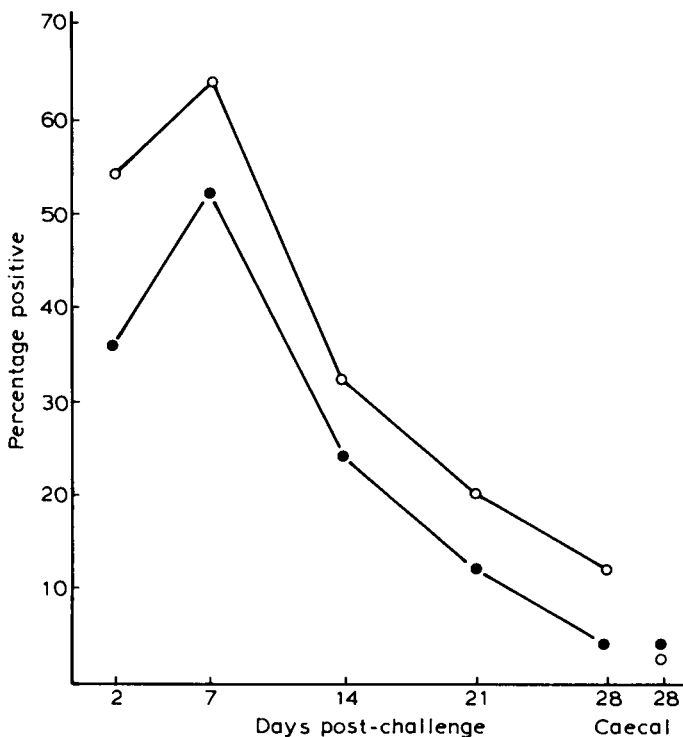


Figure 30.4 Percentage of chicks positive for *Salmonella typhimurium* following challenge with 10^6 cfu at 21 days of age. Avoparcin was started in the diet when the chicks were 16 days old: ○—○, control; ●—●, avoparcin, 10 p.p.m. (from Gustafson, Beck and Kobland, 1982, by courtesy of Paul Parey Verlag, Berlin)

creased as chicks became older. This has been thoroughly documented in the vast literature on the subject.

One of the conditions which very often sets experimental studies apart from field experience is the extraordinarily clean conditions under which laboratory tests are conducted. It follows that the rate of acquisition of enteric flora might be delayed in experimental chicks, and that this could influence the outcome of these studies. The measures to which houses are cleaned between production batches varies markedly between broiler production facilities and even between countries. In the southern USA, used litter is not removed between production batches of broilers, but in Bavaria, and elsewhere, extensive measures are often taken to ensure a somewhat aseptic environment for incoming chicks.

The effect of a septic environment

On the next series of studies, extremes in the cleanliness of litter on which the chicks were placed were included in the experimental design. Twelve separate broiler houses were washed, steamed and allowed to dry. In 6 houses, clean wooden shavings were placed to a depth of several centimetres. In the remaining 6 houses, used litter from a previous non-infection trial was brought in. *Table 30.1* shows the result of a 600-chick experiment in which avoparcin was included in the diet of one-half of the chicks. The challenge dose of salmonella at 4 days was 10^4 , 10^6 and 10^8 per bird (*Table 30.1*). In general, it may be seen that, on clean litter, the infection rate is elevated and that considerable variation was seen between groups. In an attempt to define the effect of litter hygiene, the data from all groups were combined, segregated only according to the original state of the litter and the sampling intervals.

Figure 30.5 shows that if salmonella prevalence in 300 chicks on used litter and 300 on clean litter is plotted, a clear reduction in salmonella may be seen for the first several weeks. The measure of this effect may be estimated by plotting the percentage positive for the first two sampling intervals according to the challenge dose. *Figure 30.6* shows that a dose response based on early

Table 30.1 THE EFFECT OF AVOPARCIN ON SALMONELLA SHEDDING IN CHICKS RAISED ON FRESH OR USED LITTER

Litter	Diet	House	Challenge (cfu/chick)	Percentage positive on day:						
				3	10	17	24	31	38	45
Fresh	Control	350	10^4	30	86	76	58	28	16	29
		351	10^6	88	92	80	50	46	10	34
		352	10^8	98	96	66	54	64	10	2
	Avoparcin	353	10^4	28	54	42	54	36	2	4
		354	10^6	66	84	52	48	10	6	4
		355	10^8	98	100	92	64	16	14	14
	Control	356	10^4	6	22	20	22	6	16	69
		357	10^6	24	54	48	28	12	8	2
		358	10^8	94	88	45	37	2	18	41
Used	Avoparcin	359	10^4	8	20	16	8	2	2	0
		360	10^6	30	90	49	35	24	14	19
		361	10^8	94	84	71	63	19	21	15

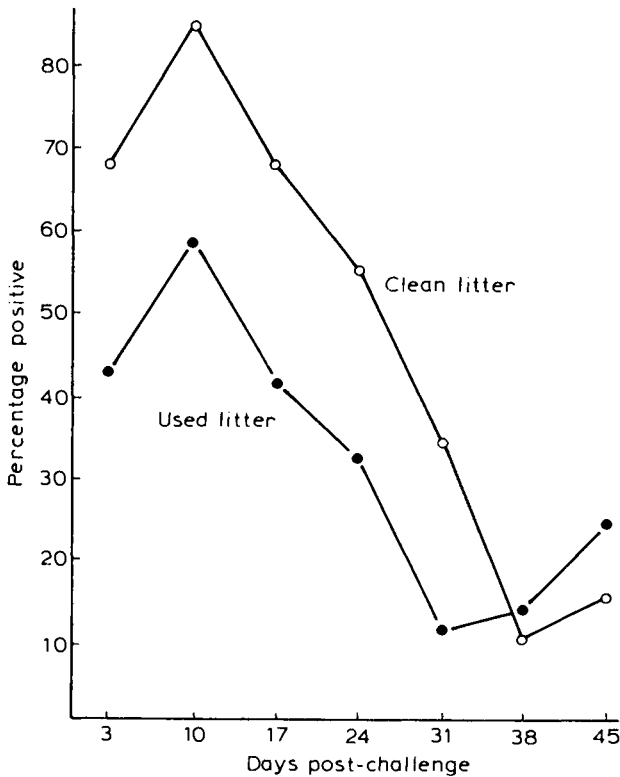


Figure 30.5 A comparison of salmonella shedding in chicks raised on clean and used litter. Data were combined from chicks challenged with 10^4 , 10^6 or 10^8 cfu and fed either control or avoparcin diets

sampling may be seen and that, on used litter, a 100-fold increase in challenge is required to achieve infection in 50% of the chicks. The median infection dose is remarkably influenced by the status of litter hygiene. This is consistent with the results of those researchers who have studied competitive exclusion as a means of controlling salmonella in broilers.

It was also possible to plot all of the data segregated on the basis of avoparcin in the diet. *Figure 30.7* shows that if all challenge levels and litter conditions are combined, there is a close agreement between chicks receiving avoparcin or a control diet. The exception is the last sampling point in which the controls showed an unexpected increase in prevalence. The reason for this is unclear, but it should probably be regarded as an aberrant result in this group of samples.

The next series of studies again examined avoparcin on clean and used litter. The model was similar to one reported by Smith and Tucker (1980) and by Jarolmen, Shirk and Langworth (1976). Whereas in these cited studies a few seeder chicks were placed with a large number of contact chicks, in this experiment, 25 birds in each house were infected with *S. typhimurium* at 10^6 , and 25 in contact with them were not infected. *Figure 30.8* demonstrates the spread of salmonella from seeder chicks to contact chicks on clean or used litter. These are combined data from avoparcin and control chicks and they

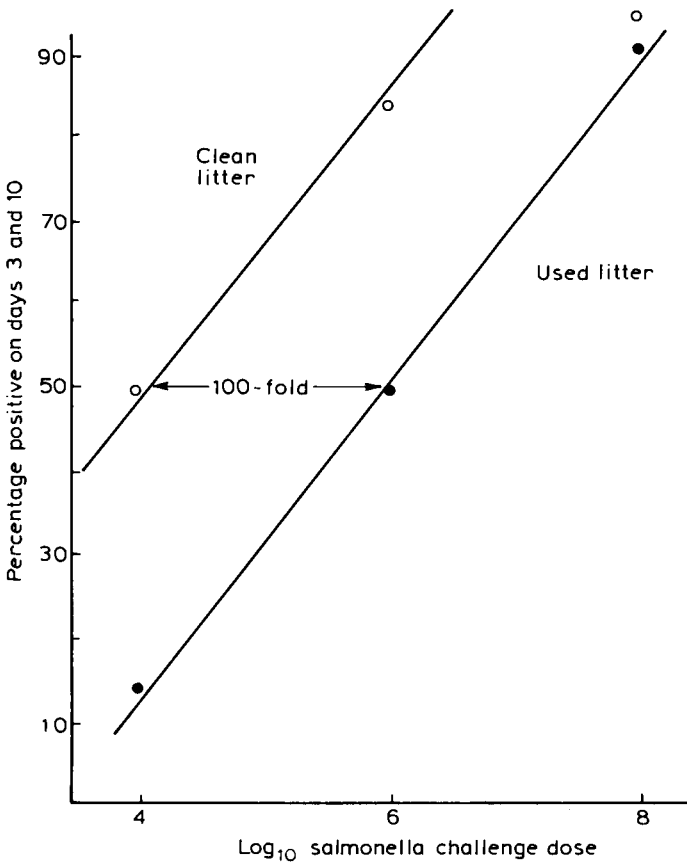


Figure 30.6 Percentage of chicks positive for *Salmonella typhimurium* on days 3 and 10 at three challenge levels and raised on clean or used litter. Data were combined from chicks receiving control or avoparcin diets

represent 150 chicks sampled per point. A reduction in the prevalence of salmonella in chicks started on used litter was confirmed. Under both conditions, symposia, public health meetings, government studies, institutional very similar after 24 or 31 days. *Figure 30.9* shows the pattern of shedding in seeder chicks and *Figure 30.10* demonstrates the acquisition of salmonella in contact chicks. On clean litter, temporary and moderate differences in salmonella were seen when avoparcin and control groups were compared but as chicks approached slaughter age, prevalence was reduced and differences disappeared. On used litter, overall infection rates were lower and no effect of feed additive could be seen.

Salmonella and its control has been the subject of many technical publications, symposia, public health meetings, government studies, institutional inquiries, media reports and communications of infinite variety. In most cases, the role of effective hygiene in the abattoir and in the kitchen has been clearly identified as a realistic measure that might be pursued with greatest

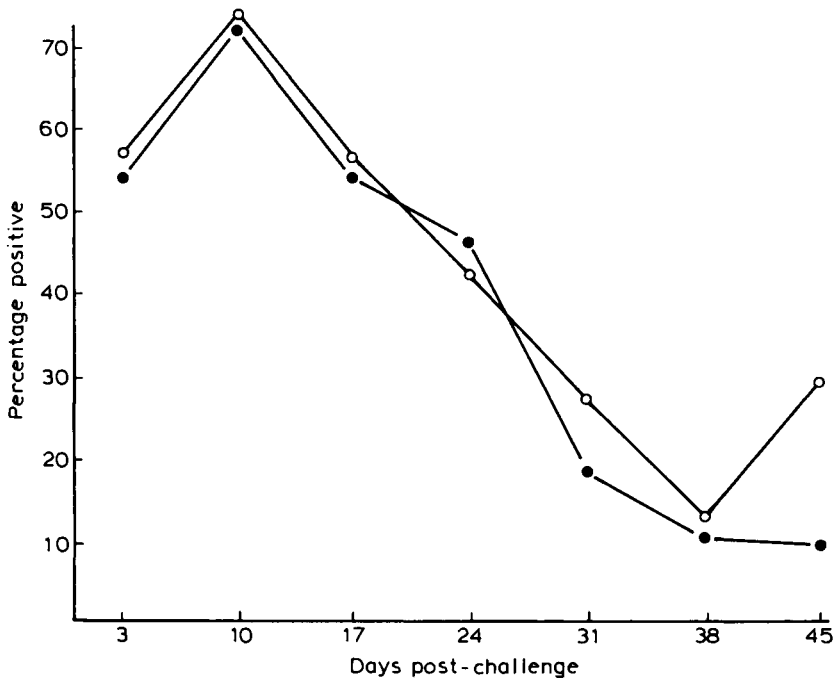


Figure 30.7 The prevalence of *Salmonella typhimurium* in chicks fed a control (○—○) or avoparcin (●—●) diet. Data were combined from 600 chicks challenged with 10^4 , 10^6 or 10^8 cfu and raised on new or used litter

effect. A protein processing order in the UK has been considered a proper step which might interrupt the flow of salmonella to animals. In some areas, the elimination of salmonella-positive birds has met with success. Our own work and the work of others leads me to believe that feed additives do not have a significant effect on the epidemiology of salmonella in broiler production. Nevertheless, the effect of feed additives on the epidemiology of this ubiquitous organism in livestock has come under review and opportunities for further studies in this area certainly do exist, especially more studies of natural infections under production conditions. It is necessary to bear in mind that controlled laboratory studies by careful investigators have led to different results in Finland, England, Germany and the USA.

The primary purpose in reviewing this subject has been to encourage researchers to be aware of the potential differences between individual experimental models and how they vary from commercial practice. Indeed, it has been observed that the prevalence of salmonella usually diminishes with time in challenge models, but the natural salmonella infections in commercial flocks often tend to remain at initial levels or increase as the broilers get older. These and other differences will inevitably lead us to a more thorough study of salmonella in livestock production and, eventually, to more effective measures for its control.

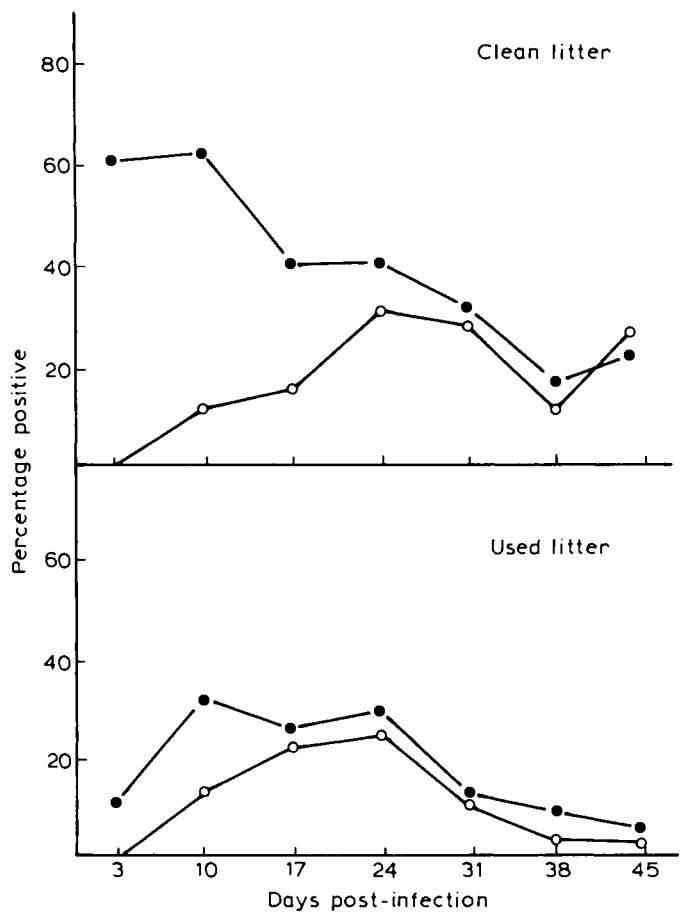


Figure 30.8 The spread of *Salmonella typhimurium* from infected to non-infected chicks raised on new or used litter. One-half of the chicks were infected with 10^6 cfu (●—●); the other half in contact with them were not infected (○—○). Data were combined from 600 chicks receiving either a control or avoparcin diet

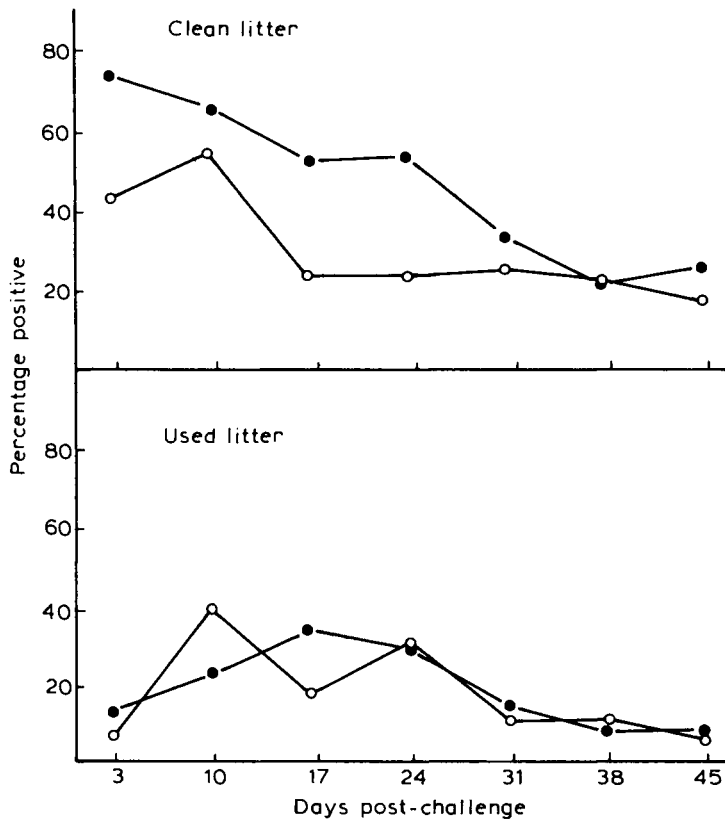


Figure 30.9 Salmonella shedding in chicks challenged with 10^6 cfu and receiving either a control diet (○—○) or avoparcin at 10 p.p.m. (●—●)

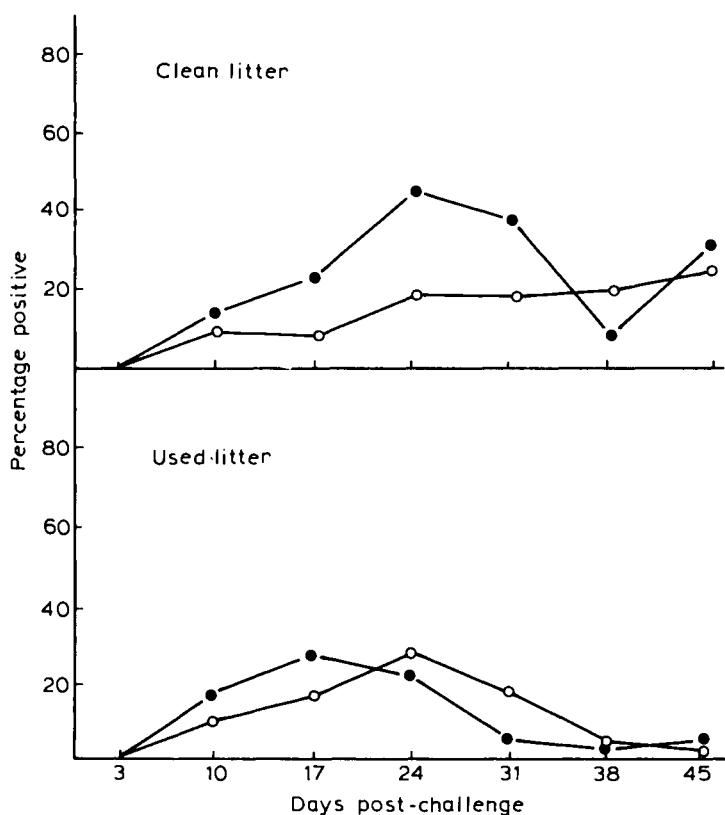


Figure 30.10 *Salmonella* shedding in non-infected chicks in contact with infected chicks and receiving either a control diet (○—○) or avoparcin at 10 p.m. (●—●)

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THE EFFECT OF SOME FEED ANTIBIOTICS ON THE MICROBIAL FLORA OF ANIMALS

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Introduction

In academic research, it is often possible to pursue a particular line of interest over many years and through many experiments. In industry, this is rarely the case. Much of industrial research is directed towards practical and immediate ends. Studies of antibiotics in animals may be for the determination of efficacy, microbiological safety or toxicity. Microbiology may therefore be an intrinsic part of these studies or it may be incidental. Over the past 15 years, the effect of a number of antibiotics on the microbial flora of animals have been studied. This presentation includes 13 separate studies in cattle, pigs, chickens or rats.

Four antibiotics were studied: apramycin, tylosin, monensin and actaplanin. The first three are currently marketed. Actaplanin was investigated as a growth promoter in pigs and chickens. Chemically, they are very different: apramycin is an aminoglycoside; tylosin a macrolide; monensin a macro-tetrolide and actaplanin a glycopeptide. Their mode of action against bacteria is also different. Apramycin and tylosin share the property of interfering with protein synthesis, but in quite distinct ways. Monensin increases cation permeability selectively and actaplanin inhibits cell-wall synthesis. Apramycin has a broad spectrum of activity but, typically for an aminoglycoside, poor activity against streptococci and none against anaerobes. Tylosin has good activity against Gram-positive and some Gram-negative bacteria, but not against Enterobacteriaceae. Monensin and actaplanin have relatively weak antibacterial activity and, for both, it is confined to Gram-positive species. They have in common a reasonable degree of stability and are either not absorbed or are excreted in bile. Therefore, when given orally, they all reach the intestine and have some possibility of affecting the normal microbial flora. All the studies are concerned with oral administration, whether for therapy or growth promotion, to healthy animals, or to those suffering from naturally acquired disease. The studies have been selected on the basis of all showing some change in the microbial flora and all being different either in the antibiotic, the dose or the animal species.

The microbiology was carried out in our own laboratory at Windlesham, apart from one study by Dr R.A. Elliott at the Lilly Laboratories in the USA,

two by Dr J.R. Walton of Liverpool University and one at the Department of Pathology, Veterinary High School, University of Madrid.

Isolation of organisms was mainly from freshly obtained faecal material, apart from the studies in chickens, where the birds were killed and the caecal contents used.

For each antibiotic, the studies are listed, together with the effects on numbers of organisms. Following a description of the individual studies, a further set of tables lists the dose which has an effect on particular groups of organisms. These effects are expressed in terms of the dose in mg/kg of bodyweight and the median minimum inhibitory concentration (MIC) of the antibiotic in mg/l. The MIC has in all cases been determined by tests of a doubling dilution series of the antibiotic in a suitable agar medium. Where there is a large scatter of MIC, including susceptible and resistant strains or species, the group of organisms has been split accordingly. From these two values, factors of dose/MIC have been calculated for increase, decrease or elimination of the particular organisms. Finally, these factors are brought together for the four antibiotics and the implications of the overall picture discussed.

Apramycin

Apramycin was studied in six experiments: three field efficacy trials in the treatment of naturally occurring diseases in pigs or calves; two experimental studies of microbiology in healthy pigs; and a sub-acute toxicity test in rats (*Table 31.1*). Apart from the toxicity test, the time of dosing was 5 days.

In the first experiment by Dr Walton, pigs weighing an average of 15 kg and being 8 weeks old were dosed with apramycin in the drinking water at the equivalent of 8 mg/kg. Faecal samples were obtained daily from the rectum of each of 10 pigs on apramycin and from 10 controls. Total viable counts of *Escherichia coli*, streptococci and lactobacilli were made and the incidence of apramycin-resistant *E. coli* determined. Numbers of *E. coli* decreased in one-half of the apramycin-treated pigs. There were very few apramycin-resistant *E. coli*, the most in any pig on any one day being 0.05%. Lactobacilli were unaffected and numbers of streptococci erratic.

The next experiment was a field trial in 12 calves on a farm at Slough. They were dosed at 20 mg/kg in capsules at the onset of naturally occurring enter-

Table 31.1 APRAMYCIN STUDIES

Dose (mg/kg)	Time (days, weeks)	Animal	Numbers of microorganisms		
			Increased	Decreased	Eliminated
8	5 d	Pig	—	<i>E. coli</i> 5/10	—
20	5 d	Calf	Yeasts 12/12	—	<i>E. coli</i> 10/12
20	5 d	Pig	Streptococci 2/8 Yeasts 3/8	—	<i>E. coli</i> 6/8
35	5 d	Pig	—	<i>E. coli</i> 10/10	<i>E. coli</i> 1/10
40	5 d	Calf	Yeasts 17/19	Streptococci 3/19	<i>E. coli</i> 19/19
500	10 w	Rat	—	Yeasts 6/8	<i>E. coli</i> 8/8 Streptococci 8/8 Yeasts 2/8

itis. Rectal swabs were obtained pre- and post-dosing, i.e. on days 1 and 6, and plated on MacConkey agar. For the trial itself, comparison was made with untreated controls, but for the present purpose, comparison is made of the appearance of organisms on the post-dosing, with that on the pre-dosing, plate. For all the calves, there was an increased proliferation of yeasts and, for most of them, elimination of *E. coli*.

Piglets treated at 20 mg/kg with an oral doser from the onset of neonatal diarrhoea were also swabbed pre- and post-dosing. They were together in litters of average size 10, but the particular animals were marked so that the same piglet could be sampled on the second occasion. As in the calves, there was some elimination of *E. coli* and increase in other organisms.

In Dr Walton's second experiment, pigs weighing an average of 15 kg and being 8 weeks old were dosed in the drinking water at the equivalent of 35 mg/kg. There was a significant reduction in the numbers of *E. coli* in the treated pigs compared with the controls, but elimination in only one of the 10 animals on the fourth day of treatment. This is less dramatic than in the piglet trial and probably indicates greater stability of the bacterial population in the older animal. A few resistant *E. coli* were found erratically in most of the treated pigs, but the number never exceeded 7.5×10^4 g in any pig on any one day. As the total *E. coli* count in that pig was 1.85×10^8 /g on the same day, there was a maximum of 0.04% of resistant strains. Numbers of lactobacilli showed no difference between treated and controls and isolation of streptococci was inconsistent. An interesting observation was that there was an irregular change in species dominance from *Streptococcus faecalis* to *S. faecium*.

The next set of results is from a field trial in Spain. One-week-old calves, weighing approximately 37 kg, were treated with apramycin in capsules at the onset of naturally occurring enteritis and/or respiratory disease. Rectal swabs were taken pre- and post-dosing and plated out. There was a marked change in the microbial flora. *Escherichia coli* was eliminated from all 19 calves and yeasts increased in 17. A few showed a decrease in the numbers of streptococci.

Finally, there is the one toxicological study in which we examined the faecal microorganisms of rats. Twenty-four animals were arranged in groups of 3 per cage. Apramycin was incorporated in the diet and the concentration adjusted to keep the treatment level at 500 mg/kg. Pooled faecal samples were taken from each cage before and after 10 weeks treatment. There were therefore 8 samples on each occasion, which were plated on MacConkey, nutrient and Sabouraud agar. A few colonies were selected for MIC determinations in each group of organisms isolated. As would be expected from the other studies, the *E. coli* were eliminated from the faeces collected in all eight cages. Streptococci were also eliminated. Yeast colonies decreased in all and were eliminated from two of the cages.

Tylosin

Tylosin is an old-established antibiotic and there have not been so many occasions to do microbiological work with it as with a newer product such as apramycin. Two quite different studies are appropriate for the present purpose (Table 31.2).

Table 31.2 TYLOSIN STUDIES

Dose (mg/kg)	Time (weeks)	Animal	Numbers of microorganisms		
			Increased	Decreased	Eliminated
4	5	Pig	Staphylococci 10/10 MIC $\geq$ 512	—	Staphylococci 10/10 MIC $\leq$ 4
10-20	Varied	Pig	Campylobacters MIC > 128	Campylobacters MIC $\leq$ 4	—

In the first study, pigs with an average weight of 35 kg were brought into the fattening house of a farm in Berkshire. Tylosin was incorporated into the feed at a dose equivalent to 4 mg/kg of bodyweight. Clinical evidence of swine enzootic pneumonia was initially present and the main purpose of the trial was to examine the efficacy of tylosin in treating this disease. The opportunity of this trial was taken to study the effect of tylosin on staphylococci. For the pre-dose samples, both nasal and rectal swabs were obtained as there was some doubt about whether we should obtain reliable recovery of these organisms from faeces. Swabs were placed in 10% salt broth and incubated overnight at 37°C. The broths were plated on nutrient agar and any morphologically apparent staphylococci purified as necessary and examined microscopically after Gram-staining. An average of 2-3 cultures was examined from each pig. As good recovery was obtained from the rectal swabs, these only were used for the subsequent samples after 5 weeks' treatment. All isolates were tested for tylosin MIC. Initially, there were 4 isolates (12%) resistant to tylosin. By 5 weeks, all 31 isolates were resistant. This is a clear case of resistant organisms being present initially which were selected by a therapeutic dose level of the antibiotic, given during a long period of treatment. Staphylococci were not relevant to the disease for which the antibiotic was being used.

The second tylosin study is the only one in the present series to have been published, although in a different form (Mackinnon, 1981). Faecal samples from live pigs, or parts of intestinal tract from dead ones, were brought to the laboratory from animals suffering from swine dysentery. Some of the pigs had been treated with tylosin and others had not and therefore acted as controls. The duration of treatment was variable and, in some cases, intermittent. Tylosin was incorporated in the feed or mixed in the drinking water. The samples were examined for what was then called *Vibrio coli*, but which would now be described as *Campylobacter* spp., probably mainly, if not entirely, *C. coli*. The isolation method was that of Fletcher and Bertschinger (1964) and the growth medium tryptone blood agar and blood agar slopes to which tryptone broth had been added (G.H.K. Lawson, 1966, personal communication). Inoculated media were placed in an atmosphere containing 10% carbon dioxide and incubated at 37°C for 3-6 days. Colonies were examined for characteristic morphology and the bacteria viewed under the microscope. Tylosin MIC was determined for each isolate. For the present purpose, comparison is made between the tylosin susceptibility of isolates from the treated and the untreated controls. The results show a tendency for the selection of resistant *Campylobacter* spp. from the treated pigs, but some susceptible strains were still present.

These studies on tylosin complement each other very well. At first sight, the higher dose range has a reduced effect, but two points need to be noted.

In the *Campylobacter* study, even though the time of dosing is variable, it was never as much as 5 weeks. Decrease in numbers may lead to elimination if the time of dosing is prolonged. Also, in this study, it is not safe to assume that failure to isolate was a proof of elimination. All the samples had to be brought to the laboratory from outside sources, some at a considerable distance. The state of the sample and the speed with which it could be used had a considerable effect on the success in isolating rather delicate bacteria. So, any samples from which the organisms were not isolated have been omitted. The tylosin MIC range for susceptible staphylococci and *Campylobacter* spp. overlapped, but the median values were 2 and 4 mg/l, respectively.

Monensin

There are three studies on monensin, the first being at two levels (Table 31.3). Monensin was included in the starter and finisher feeds of individually housed veal calves for a period of 12 weeks. This was a trial designed to evaluate the effect of monensin on growth performance. As it was on our own site, there was easy access to the animals to investigate the effect of monensin on *E. coli* and streptococci. Faecal samples were obtained weekly from the 6 calves in each of the treated groups and from 6 untreated controls. Viable counts of both groups of organisms were carried out and 2–3 colonies of each selected for MIC determinations. The *E. coli* were uniformly resistant to monensin and there was no change in their numbers at either dose, in comparison with the controls. The streptococci showed a slight reduction in numbers in the treated animals by the end of the period. These streptococci were susceptible to monensin, but there was a wide range of MIC from <0.015 to 8 mg/l. For this presentation, they have been divided into three groups: <0.125, 0.25–1.0 and 2–8 mg/l. The proportion in the lowest group was reduced by a dose of 0.35 mg/kg and in both lower groups by a dose of 0.7 mg/kg. These changes were apparent by the sixth week and were slightly more marked by the twelfth week. Examination of some of these organisms showed that those least susceptible to monensin were also penicillin resistant. Diagnostic tests showed that these bacteria were *S. faecium*, which is more resistant than *S. faecalis*, the other common faecal streptococcus. So, the effect of monensin was to increase the proportion of a less susceptible species but, even after 12 weeks, not to eliminate the more susceptible.

Table 31.3 MONENSIN STUDIES

Dose (mg/kg)	Time (weeks)	Animal	Numbers of microorganisms		
			Increased	Decreased	Eliminated
0.35	1–12	Calf	Streptococci MIC 2–8	Streptococci MIC $\leq$ 0.125	—
0.7			Streptococci MIC 2–8	Streptococci MIC $\leq$ 1.0	—
0.9	8	Calf	Bacteroides	—	—
4	4	Chicken	Lactobacilli MIC > 128 Bacteroides	Lactobacilli MIC 0.125–0.5	Streptococci MIC 0.125–0.5

In the next study, also on our own site, calves initially of 16–23 weeks of age were fed monensin in the starter ration for 8 weeks. This trial was designed to test the effect of monensin on numbers of faecal bacteroides. Viable counts were carried out on samples taken at approximately 10-day intervals from 6 treated and 6 control animals. The selective medium was Blood Agar Base No. 2 (Oxoid CM 271), with addition of neomycin, 100 mg/l, and vancomycin, 7.5 mg/l. Plates were incubated under 90% hydrogen/10% carbon dioxide. At each time interval, one colony was selected from each animal for MIC determination. The mean count in both groups increased during the first test period, significantly more in the monensin group than in the controls. The difference between the group counts remained significant for the rest of the trial. The control counts remained steady, while the counts from the treated calves appeared to rise slightly, but not significantly. Monensin MIC results were all ≥ 128 mg/l.

The final monensin study was in chickens at the level of antibiotic originally used for control of coccidiosis. Monensin was incorporated in the feed for 4 weeks of birds initially 8 weeks of age. Five treated and 5 control birds were killed at the end of treatment and the caecal contents obtained. These were diluted and plated on appropriate media for the enumeration of *E. coli*, streptococci, bacteroides and lactobacilli. Determinations of MICs were carried out on a few isolates of each. There was no effect of monensin on the numbers of *E. coli* or lactobacilli, but there was an increase in the proportion of resistant lactobacilli. Bacteroides increased from 10^8 /g to 10^9 /g and, most notably, streptococci were eliminated. In the controls, all the streptococci tested were in the MIC range 0.125–0.5 mg/l.

Actaplanin

Actaplanin was studied in two experiments: one in chickens and the other in pigs (Table 31.4). The first of these, at Windlesham, was similar to the final monensin study, but younger birds were used. One-day-old chickens were obtained and divided into 3 groups, 2 levels of actaplanin and controls. Birds were killed in pairs from each group when aged 1, 4 and 7 weeks. The caecal contents were diluted and plated on media for the enumeration of *E. coli*, streptococci, bacteroides, lactobacilli and clostridia. The MIC of actaplanin was determined for at least one isolate in each group of organisms for each bird. *Escherichia coli* were resistant to actaplanin and showed no effect of the antibiotic on numbers. Most lactobacilli were also resistant and showed a decline in numbers in both control and treated groups. Clostridia were of variable susceptibility and the numbers were erratic. Bacteroides were resist-

Table 31.4 ACTAPLANIN STUDIES

Dose (mg/kg)	Time (weeks)	Animal	Numbers of microorganisms		
			Increased	Decreased	Eliminated
0.5	7	Chicken	Bacteroides	Streptococci	—
1.0			Bacteroides	Streptococci (4 w)	Streptococci (7 w)
2.5	12	Pig	<i>E. coli</i>	—	Clostridia (6 w)
25.0			<i>E. coli</i>	—	Clostridia (4 w)

ant and increased in both treated groups compared with the controls. As with monensin, the most striking differences were in the streptococci. All were susceptible to actaplanin at 4 mg/l. At 0.5 mg/kg, there was a decrease from 10^7 to 10^6 and at 1.0 mg/kg they became undetectable, i.e. $<10^3$ /g, the sensitivity limit of the method.

The second study, at the Lilly laboratories in Greenfield, Indiana, examined the effect of actaplanin on the faecal *E. coli* and *Clostridium perfringens* of pigs. Three groups of 4 pigs, initially 10 weeks of age, were treated for 12 weeks with actaplanin in the feed, at one of two levels or negative controls. In order to promote the reliable presence of clostridia, 10% meat scraps formed part of the feed. Faecal samples were taken at 2-week intervals and the numbers of *E. coli* and *C. perfringens* determined on MacConkey agar and TSN agar (BBL), respectively. Actaplanin MIC was determined on representative isolates at each time interval. The numbers of *E. coli* increased significantly in the actaplanin treated groups from 10^6 /g to 10^8 /g, compared with the controls. In the lower treatment group, this change was apparent after 4 weeks and in the higher group by 2 weeks. Thereafter, the numbers in the actaplanin treated groups remained steady, but there was a slight fall to 10^5 /g in the controls. The numbers of *C. perfringens* remained steady in the controls at 10^6 – 10^7 /g, but dropped rapidly in the treated groups to 10^2 – 10^3 /g at 2 weeks. They became undetectable, apart from occasional isolates, i.e. $<10^2$ /g, in the 2.5 mg/kg group by 6 weeks and in the 25 mg/kg group by 4 weeks. There was no development of resistant clostridia.

Summary and discussion

The effect of each of these antibiotics is shown in *Tables 31.5–31.8*, in which the affected organisms are listed against the dose which increased, decreased or eliminated them. At the base of each table, three values of the factor, dose/

Table 31.5 DOSE OF APRAMYCIN AFFECTING PARTICULAR MICRO-ORGANISMS

Microorganisms	Median MIC (mg/l)	Dose (mg/kg)		
		Increased	Decreased	Eliminated
<i>E. coli</i>	8	—	8	40
Streptococci	128	20	40	500
Yeasts	≥ 512	20	500	—
Dose/MIC		≤ 0.16	≥ 0.3	≥ 4

Table 31.6 DOSE OF TYLOSIN AFFECTING PARTICULAR MICROORGANISMS

Microorganisms	Median MIC (mg/l)	Dose (mg/kg)		
		Increased	Decreased	Eliminated
Staphylococci	2	—	—	4
	> 512	4	—	—
<i>Campylobacter</i> spp.	4	—	10–20	—
	> 128	10–20	—	—
Dose/MIC		≤ 0.16	≥ 2	≥ 2

Table 31.7 DOSE OF MONENSIN AFFECTING PARTICULAR MICRO-ORGANISMS

<i>Microorganisms</i>	<i>Median MIC (mg/l)</i>	<i>Increased</i>	<i>Dose (mg/kg)</i> <i>Decreased</i>	<i>Eliminated</i>
Streptococci	≤ 0.125	—	0.35	4
	0.25–1.0	—	0.7	4
	2–8	0.35	—	—
Lactobacilli	0.125–0.5	—	4	—
Bacteroides	> 128	4	—	—
	≥ 128	0.9	—	—
Dose/MIC		≤ 0.17	≥ 0.7	≥ 4

MIC, are given. For organisms which have increased, it is the highest value and for those which have decreased or been eliminated, the lowest value that is applicable. For this purpose, organisms are counted as having increased or decreased if this has occurred in some, but not necessarily all, in a group of animals. Organisms are counted as having been eliminated only if this is true of the entire group of animals.

Finally, the values of dose/MIC for all four antibiotics are summarized

Table 31.8 DOSE OF ACTAPLANIN AFFECTING PARTICULAR MICRO-ORGANISMS

<i>Microorganisms</i>	<i>Median MIC (mg/l)</i>	<i>Increased</i>	<i>Dose (mg/kg)</i> <i>Decreased</i>	<i>Eliminated</i>
<i>C. perfringens</i>	0.25	—	—	2.5 (6 w) 2.5 (4 w)
Streptococci	1.0	—	0.5 (7 w) 1.0 (4 w)	—
<i>E. coli</i>	> 128	2.5	—	—
Bacteroides	> 512	0.5	—	—
Dose/MIC		≤ 0.02	> 0.5	≥ 10

(Table 31.9). This shows a narrow range of variation between the antibiotics and suggests that these results might have wider application. It may be possible to predict how other compounds would affect the intestinal flora. For example, an antibiotic given as a growth promoter at 1.0 mg/kg is likely to eliminate organisms with MIC 0.125 mg/l, at least reduce those with MIC 1.0 and may allow any with MIC 16 or above to increase. An increase in numbers of any group of organisms is only likely to occur if some other organisms are reduced. It could also be predicted that an antibiotic with therapeutic application given at 20 mg/kg is likely to eliminate organisms with MIC 2 mg/l, at least reduce those with MIC 16 and permit the increase of any with MIC 256 or above. There would be no expected effect on organisms with MIC 32 and therefore these could be considered resistant. Resistance *in vivo* is often difficult to define and this method gives a practical way of doing it and overcoming the difficulty.

Another question that is sometimes posed is whether the use of an antibiotic at a low level as a growth promoter will prejudice its therapeutic use at a higher level. For tylosin as a growth promoter at 1–2 mg/kg, it could be

Table 31.9 SUMMARY OF ANTIBIOTIC EFFECTS OF MICROORGANISMS

Antibiotic	Dose (mg/kg)/MIC (mg/l)		
	Increased	Decreased	Eliminated
Apramycin	≤ 0.16	≥ 0.3	≥ 4
Tylosin	≤ 0.16	≥ 2	≥ 2
Monensin	≤ 0.17	≥ 0.7	≥ 4
Actaplanin	≤ 0.02	> 0.5	≥ 10
Dose/MIC			
Mean (range)	0.13 (0.02-0.17)	0.9 (0.3-2)	5 (2-10)

predicted that organisms, such as the susceptible *Campylobacter* spp., with MIC 4 mg/l would be unaffected.

Orally administered antibiotics are diluted and then concentrated, as they pass through the gut, but the extent of both processes is generally unknown and assay is difficult. Even if the concentrations are known, there is no necessary relationship between the inhibitory concentration of the antibiotic for a pure culture on an agar plate and that in the very different environment of the intestine. The prediction from the figures presented here depends on values that are known from the dose given, the weight of the animal and the *in vitro* MIC.

In conclusion, it is suggested that researchers should test these predictions on their own data and see if these factors, or something like them, are applicable.

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THE ROLE OF STREPTOCOCCUS FAECIUM IN ANTIBIOTIC-RELIEVED GROWTH DEPRESSION OF CHICKENS

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The original observation (Moore *et al.*, 1946) that streptomycin stimulated the growth of chicks was soon followed by many more reports of the growth-promoting effects of antibiotics and other antibacterial compounds (see Luckey, 1959) including penicillin (Coates *et al.*, 1951). The common antibacterial nature of all these supplements suggested early on that the effect was being mediated through the bacteria populating the intestinal tract. Subsequently, it was demonstrated that in the absence of the gut microflora (i.e. in germ-free chickens) dietary penicillin had no effect on growth (Coates *et al.*, 1963). Thus, it was concluded that growth stimulation of poultry by dietary antibiotics was dependent on the existence in the gut of a population of bacteria which adversely affected growth. In the past *Clostridium perfringens* (Sieburth *et al.*, 1951; Lev, Briggs and Coates, 1956) and *Streptococcus faecalis* (Huhtanen and Pensack, 1965; Eyssen and De Somer, 1967) were claimed to be responsible for the growth-depressing effect. More recently, *S. faecium* has been put forward as a growth-depressing agent (Fuller, Coates and Harrison, 1979). The evidence for this last organism as a causal agent of growth depression and a discussion of the possible mechanism involved follows.

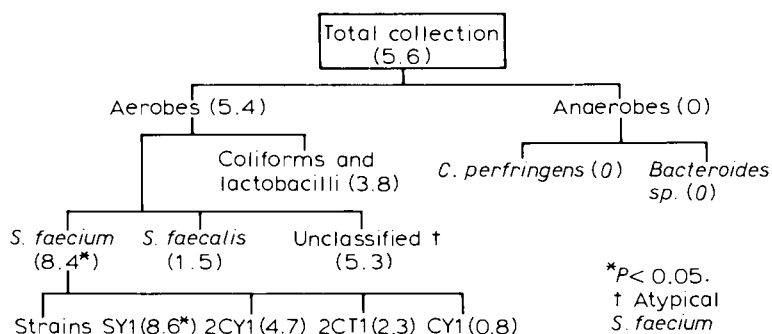


Figure 32.1 Growth depression produced by re-establishing groups of bacteria in the intestines of gnotobiotic chickens. Figures in parentheses show growth depression as a percentage of germ-free weight (after Fuller *et al.*, 1981)

Identification of *Streptococcus faecium* as a growth-depressing organism

A collection of organisms isolated from the crops and caeca of conventional chicks whose growth was depressed was fed to germ-free chicks. The size of the resulting growth depression was comparable to the growth stimulation observed in conventional chicks fed penicillin supplements in the diet. The identification of the growth-depressing organism was achieved by separating the collection into groups of related organisms. These groups were administered to germ-free chicks and the effect on growth determined at 2 weeks of age. The active group was then further fractionated into single species and finally into single isolates. The results are shown in Figure 32.1. The growth-depressing activity was confined to the streptococcus group and when this was further examined it was found that *S. faecium* was the active species. *Streptococcus faecalis* and two unclassifiable streptococci failed to depress growth significantly. Within the *S. faecium* group, the amount of growth depression produced by individual strains was variable. The most active growth-depressing strain was *S. faecium* strain SY1 and all subsequent work was done with this isolate.

Effect of penicillin on *Streptococcus faecium*

During the first week after hatching, the count of *S. faecium* in the small intestine reaches a high concentration (c. 10^7 cfu/g) and then declines during the second week of life. The counts in the crop are lower and do not increase (Figure 32.2; Houghton and Fuller, 1980). This increase in count in the duodenum indicates that growth is taking place in that region. The effect of penicillin was studied by rearing chicks on diets with and without the antibiotic and examining the *S. faecium* population in the gut at 3 days after hatching. Dietary penicillin reduced the count of *S. faecium* throughout the intestinal tract and prevented the multiplication of *S. faecium* in the duodenum (Houghton, Fuller and Coates, 1981). This inhibition in the duodenum was related to the growth stimulation produced by penicillin. If the mean

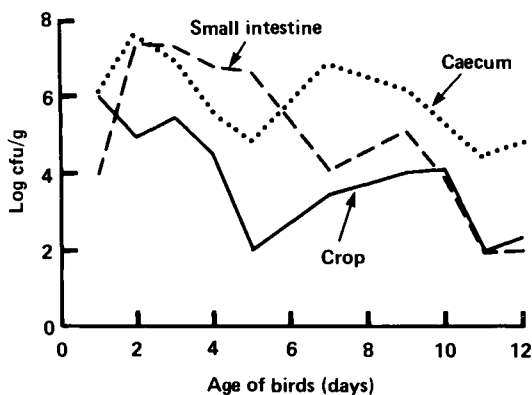


Figure 32.2 Viable counts of *Streptococcus faecium* in the intestinal tract of chickens up to 12 days after hatching (from Fuller *et al.*, 1981)

Table 32.1 GROWTH DEPRESSION OF CHICKS WITH AND WITHOUT *Streptococcus faecium* GROWING IN THE DUODENUM

<i>S. faecium</i> in duodenum	No. of experiments	Viable count ^a of <i>S. faecium</i> Crop	<i>S. faecium</i> Duodenum	Growth response to penicillin (% of control)
Growth	16	4.0	5.8	6.4
No growth	8	5.6	3.4	1.3

^a Log₁₀ cfu/g wet weight.

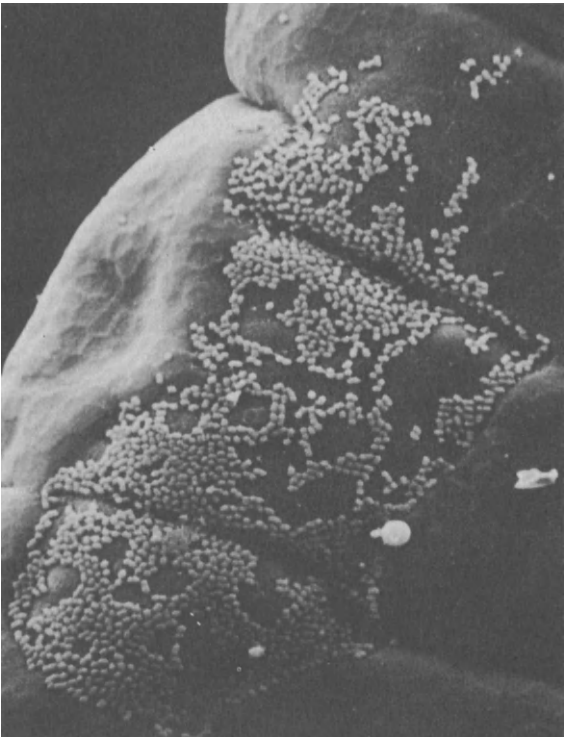
growth stimulation for groups showing duodenal growth is compared with that for groups where there was no increase in numbers of *S. faecium* in the duodenum, the respective values are 6.4% and 1.3% (Table 32.1).

Similar studies were made with gnotobiotic chicks mono-associated with *S. faecium* and fed penicillin (Fuller, Houghton and Coates, 1983). In the control birds, *S. faecium* grew to large numbers in the duodenum and depressed chick growth compared with germ-free chicks. When penicillin was included in the diet, the numbers of *S. faecium* in the duodenum decreased and the growth depression was relieved.

Importance of epithelial attachment

The increased count of *S. faecium* in the duodenum compared with that in the crop indicated that growth was taking place in the upper small intestine, in spite of the rapid passage of chyle through this part of the gut. One way in which intestinal organisms can counteract the flushing effect of peristalsis is to attach to the gut wall (see Fuller and Brooker, 1980). Evidence that this was occurring with *S. faecium* in the chick gut was sought by culture of washed gut wall and by electron microscopy. Viable counts of *S. faecium* in washed macerated duodenal tissue which exceeded the count of the last washing solution indicated that *S. faecium* was attached to the duodenal epithelial surface (Fuller, Houghton and Brooker, 1981). This close association of *S. faecium* with the epithelial surface was confirmed by scanning and transmission electron microscopy (Figure 32.3a and 32.3b). The transmission photographs revealed a lucent zone surrounding the streptococcus cells which was due to unstained capsular material. The scanning studies showed a patchy distribution of *S. faecium* on the gut surface. This uneven distribution could be due to receptors occurring in patches or due to receptors being obscured by mucus. The latter seems more likely because when suspensions of washed duodenal brush borders were tested, attachment occurred on virtually all of the brush borders.

Although attachment occurred with washed brush borders, the number of *S. faecium* attached per brush border could be increased by adding the washings produced during preparation of the brush borders back to the adhesion test system. Subsequently, it was shown that the factor responsible for the enhanced adhesion was trypsin (Fuller, Houghton and Brooker, 1981). Thus, adhesion could be increased by adding trypsin and the adhesion-enhancing effect of washings could be prevented by soya bean trypsin inhibitor (Table 32.2). The possibility that this trypsin-dependent adhesion was



(a)

Figure 32.3 *Streptococcus faecium* attached to duodenal epithelium of the chicken: (a) scanning electron micrograph showing uneven distribution of the bacterial cells on the surface of the villus; (b) transmission electron micrograph showing bacteria surrounded by lucent zone indicating capsular material. Note distortion of microvilli adjacent to the attached bacterial cells (from Fuller *et al.*, 1981)

(b)

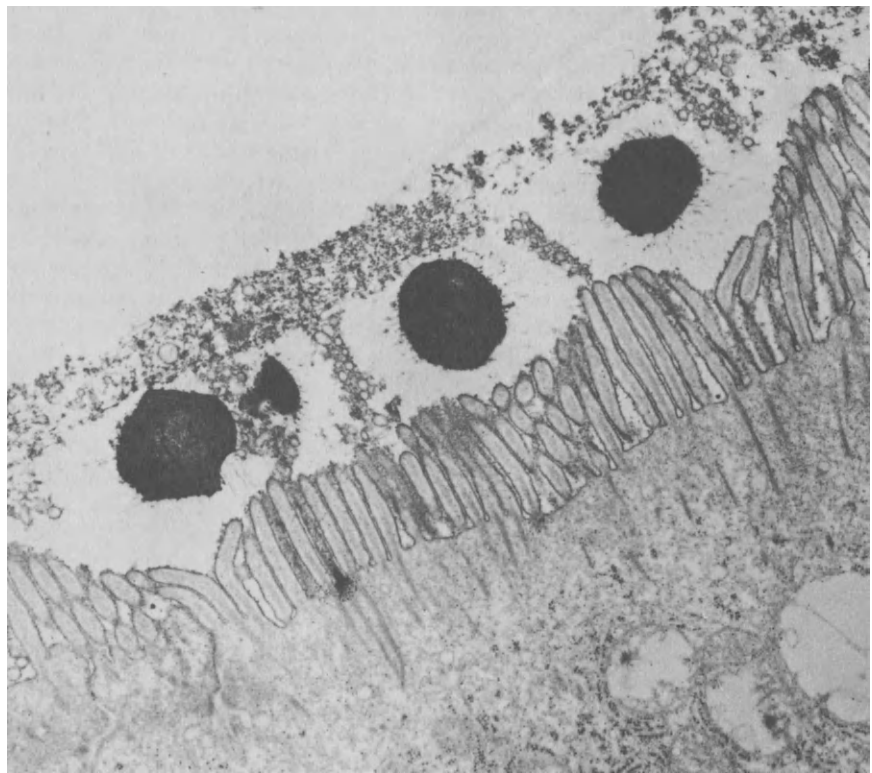


Table 32.2 ENHANCING EFFECT OF TRYPSIN ON THE ADHESION OF *Streptococcus faecium* TO DUODENAL BRUSH BORDERS

Treatment	Adhesion of <i>S. faecium</i> ^a
None	7.2
Duodenal washings	15.8
Duodenal washings + SBTI ^b	7.9
Trypsin	15.4
Trypsin + SBTI	6.1

Source: from Fuller *et al.*, 1981.

^a Mean number of bacteria/brush border.

^b Soya bean trypsin inhibitor.

inactivating a significant amount of trypsin and thereby depressing growth was discounted because it was not possible to demonstrate a detectable loss of tryptic activity when *S. faecium* was attached to brush borders *in vitro* in the presence of trypsin.

Bile acid deconjugation

The two main bile acids in chicken bile are taurocholic acid and taurochenodeoxycholic acid (Haslewood, 1967). *S. faecium* is capable of deconjugating both these acids to form the free bile acids. Many of the other bacteria indigenous to the chicken gut can also deconjugate bile acids, but the only other organisms which can deconjugate bile acids and attach to the gut epithelium are some of the lactobacilli (Cole and Fuller, unpublished data). However, they are not growth depressing. It may be that the *in vitro* activity is not manifested *in vivo*. Dickinson, Gustafsson and Norman (1971) found that with the strains of lactobacilli which they tested, the activity demonstrable *in vitro* was not found when the strains were established in gnotobiotic rats.

Effect on nutrition

Studies using isolated loops of the small intestine in anaesthetized chicks indicate that the presence of a gut flora does not affect absorption of glucose (Coates *et al.*, 1981) or methionine (Yokota and Coates, 1982). However, a lipid balance experiment showed that more lipid was excreted in conventional than in germ-free chicks (Cole, Fuller and Coates, 1981). There were no differences between the two groups in digestibility of saturated and unsaturated fatty acids. This contrasts with the findings of Boyd and Edwards (1967), who found that germ-free chicks retained greater amounts of palmitic and stearic acids than did conventional chicks. In our experiments, the difference in total lipid excretion between the germ-free and conventional chicks was statistically significant, but whether it is sufficient to account for the depression of growth is as yet unknown.

Proposed mechanism

On the basis of the evidence available, the following mechanism is proposed for antibiotic-relieved growth depression of chickens (Figure 32.4). The causative organism, *S. faecium*, is able to adhere to the duodenal surface and as a result can survive in large numbers and multiply during the first week after hatching. This population of *S. faecium* deconjugates bile acids and has an

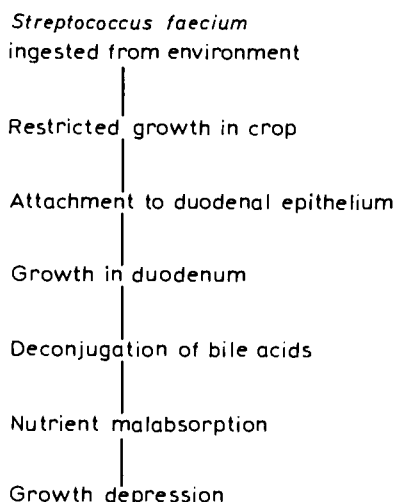


Figure 32.4 Proposed mechanism for growth depression of chicks by *Streptococcus faecium*

adverse effect on nutrient absorption and as a result growth is depressed. This suggested mechanism for antibiotic growth stimulation is very similar to that proposed for contaminated small bowel syndrome of the human infant. In this condition, stasis allows bacteria, which normally are restricted to the lower gut, to colonize the small intestine (Tabaqchali, 1970; Gracey *et al.*, 1971). Moreover, malabsorption has been related to bile acid deconjugation (Gracey *et al.*, 1971) and streptococci isolated from human infants can cause malabsorption in rat gut loops (Gracey *et al.*, 1975).

General discussion

The first organism alleged to be responsible for growth depression of chicks was *C. perfringens*. Sieburth *et al.* (1951) showed that the numbers of clostridia were reduced in penicillin-fed turkey poults and subsequently Lev and Forbes (1959) depressed the growth of germ-free chicks by administering pure cultures of *C. perfringens*. Other workers (Eyssen and De Somer, 1967; Powell *et al.*, 1974; Fuller, Coates and Harrison, 1979) were unable to reproduce the growth-depressing effect of *C. perfringens* for gnotobiotic chicks.

Streptococcus faecalis as the cause of growth depression was first suggested by Huhtanen and Pensack (1965). This finding was confirmed by Eyssen and De Somer (1967). However, prior to these observations Lev and Forbes

(1959) had not been able to produce a growth depression with *S. faecalis* subsp. *liquefaciens* and later Harrison and Coates (1972) also got normal growth in chicks colonized with this species. The findings with *S. faecium* appeared to confuse the picture even more, but the organism used by Huhtanen and Pensack (1965) and by Eyssen and De Somer (1967) may have been *S. faecium* and not *S. faecalis*. The distinction between these two species was difficult and the published characteristics would apply to either. The other two studies with *S. faecalis* (Lev and Forbes, 1959; Harrison and Coates, 1972) were done with strains that liquefied gelatin and thus could not have been *S. faecium*. Recent studies (Jeffries, Coleman and Bunyan, 1977; Barnes *et al.*, 1978), comparing the counts of *S. faecalis* in control and antibiotic-supplemented chicks, have found reduced counts of *S. faecalis* in chicks fed penicillin or bacitracin and conclude that *S. faecalis* is responsible for growth depression. In both cases the *S. faecalis* count of the anterior gut was so low ($< 10^4$ cfu/g) that it seems unlikely to be growing, metabolizing and affecting the growth of the chick. Moreover, chlortetracycline included in the diet caused an increase in the count of *S. faecalis* (Barnes, 1958). Although these experiments were primarily concerned with *S. faecium*, the use of TlTg (triphenyltetrazoliumchloride, thallous acetate and glucose) medium of Barnes (1956) enabled an assessment of the numbers of *S. faecalis* as well as *S. faecium* to be made. This showed that growth depression (as judged by the presence of a growth response to penicillin) was obtained in the absence of *S. faecalis*. The count of *S. faecium* rises and falls during the first week (Houghton and Fuller, 1980), so that if significant changes in the *S. faecium* flora are to be detected counts should be made during this period. The concept of an early peak in the effect of antibiotics was first demonstrated by Eyssen and De Somer (1963) who showed that growth depression reversed by virginiamycin or penicillin reached a maximum between the fifth and ninth day.

Although these results showed no consistent correlation between presence of *S. faecium* and the growth response to penicillin, the capacity of *S. faecium* to depress the growth of germ-free birds does show that it is potentially capable of depressing growth under conventional conditions. This finding is consistent with other findings in conventional chicks (Houghton, Fuller and Coates, 1981). The variation in penicillin growth response, even when *S. faecium* can be demonstrated in the gut, may be due to the presence on some occasions of a non-attaching strain of *S. faecium* which, as has been shown, does not colonize the gut as effectively as an adhering strain (Fuller, Houghton and Brooker, 1981) or to the presence of a penicillin-resistant strain of *S. faecium*. The pre-treatment and the environment of the *S. faecium* cells before gut colonization may also be important. Thus, it has been shown that anaerobic growth increases the growth-depressing potential of *S. faecium* strain SY1 (Houghton, Fuller and Coates, 1981).

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STAKHANOVISM* AND SIDE-EFFECTS

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Introduction

The objectives of this chapter are to discuss the consequences of feeding antibiotics as nutritional additives and to discuss the increasing attention being paid to reports of undesirable effects of antibiotic additives.

It is now over 40 years since antibiotics were first used in clinical practice and over 20 years since multiple infectious drug resistance was reported by Ochiai *et al.* (1959). In the years since then, many individuals and groups of experts have sought to achieve three answers:

- (1) What is the extent of antibiotic-associated problems in man and livestock?
- (2) Are the problems preventable?
- (3) What rules are needed to minimize the problems and to monitor the success, or not, of the control measures.

The present author has no special insight which would allow the easy solution of these questions, but an attempt has been made to summarize various contributory problems and to quantify their significance.

In order to avoid excessive repetition of the well-documented problems of infectious drug resistance, this paper includes consideration of other consequences arising from the use of animal feeds as the means of delivery for antibiotics. These will be discussed under the following headings: consequences of feeding, or not, to target species; consequences to non-target livestock; consequences to operators; consequences to consumers; interactions with other drugs; prevention of problems.

Consequences to target species

The simplest example of the need for medicating a group of animals would be when a life-threatening infection is present. It would be totally unreasonable and unethical to withhold any necessary antibiotics from such animals and, in many cases, the medication of feed will provide the most convenient means of

* *Stakhanovism*—exceptional increase in output (f. Stakhanov, a Russian miner) (*The Concise Oxford Dictionary*).

administration. At the other extreme is the debate on use of in-feed antibiotics for growth promotion. An editorial in the *Veterinary Record* (Anon., 1968) commented on the need for guidance on the epidemiology of transferable drug resistance and asked that responsible use of antibiotics should be the aim of such guidance rather than the mere allocation of guilt. The report of the Swann (1969) Committee has been thoroughly debated, although it must regretfully be said that allocation of guilt to either the farmer, the veterinarian or the medical profession has often been the objective of such debates.

The many companies still currently involved in the production of antibiotic feed additives offer a testimonial to the value which producers believe they obtain from feeding such additives, and it would be impossible to detail here

Table 33.1 INCREASED PROFIT (£) PER BACON PIG FROM USE OF GROWTH PROMOTERS

FCR improvement (%)	Additive cost per tonne (£) ^a		
	0.50	1.00	1.50
5	1.11	0.99	0.88
10	2.34	2.23	2.12
15	3.57	3.47	3.36

Source: from Hardy (1978), by courtesy of *Pig Farming*.

^a Food cost/t assumed to be £100.

all the economic benefits generated in this way. However, it must be clearly stated that the economic benefits do not reside entirely with producer or pharmaceutical companies, there being considerable advantages to the consumer in terms of food produced at a lower unit cost than would be possible without the use of performance-enhancing drugs. As an example of the economics involved, *Table 33.1* highlights the extra profit per pig when any growth promoter is used to improve feed conversion ratio (FCR) by 5–15%.

The establishment of national and European legislation in respect of the quality, safety and efficacy of antibiotics in feed is probably the most regulated area of veterinary medication. Within the UK, the Medicines Act and its subsequent Orders impose an independent, expert review of all new feed additives, their inclusion rates and the precautions necessary when using them. Thus, adverse consequences to feeding approved antibiotic additives are few in the target species.

Consequences to non-target livestock

Different species have different sensitivities to the effects of many drugs. In a review of species difference in toxicology, Clarke (1976) listed many reasons why such differences exist. These include physiological, anatomical and biochemical variations between animals, and to this list should be added management variations. The use of different antibacterials by routes other than feed must not be ignored either, since it has been demonstrated that intravenous administration of tetracyclines, streptomycin and neomycin can produce some degree of neuromuscular blockade in pigs and lambs, whereas penicillin G, chloramphenicol and sulphadiazine has no effect (Mathew *et al.*, 1978).

Out of a total of 111 adverse reactions to anti-infective agents reported by the Bureau of Veterinary Medicine (1981), in the USA, 299 deaths were reported in farm animals (*Table 33.2*). The use of products not approved for animal use or in non-target species accounts for 54 of the 185 deaths from oral administration, the remaining 131 deaths being due to feeding monensin at greater than recommended levels to cattle.

A report by Muylle *et al.* (1981) confirmed the acute toxicity of monensin to horses and described a drug-associated delayed toxicity which presented as a degenerative cardiomyopathy. Other reports have shown that particular species, or even different production types within a species, have marked sensitivities to certain drugs. Crossman and Poyser (1981) reported that

Table 33.2 DEATHS FROM ANTI-INFECTIVE AGENTS IN FARM ANIMALS IN THE USA (1979-80)

<i>Species</i>	<i>Drug</i>	<i>No. deaths</i>	<i>Approved drug</i>	<i>Route of administration</i>
Equine	Lincomycin	2	No	Oral
	Monensin	13	No	Oral
	Procaine penicillin and dihydro-streptomycin	1	Yes	Injection
	Tylosin	4	No	Injection
Bovine	Monensin	131	Yes	Oral
	Nitrofurazone	21	No	Oral
	Oxytetracycline	56	Yes	I/M
	Potassium penicillin G	1	No	Via foramen magnum
	Procaine penicillin G	7	Yes	Injection
	Benzathine penicillin	7	Yes	Injection
	Procaine penicillin and dihydrostreptomycin	26	Yes	Injection
	Spectinomycin	12	No	Injection
	Sulphamethazine	2	Yes	Oral
Porcine	Monensin	16	No	Oral

tylosin and tylosin + dimetridazole have detrimental effects in the dairy cow, but not in heifers, steers or bulls. Dimetridazole and lincomycin were similarly included accidentally in dairy cattle cake and symptoms of diarrhoea and recumbency were noted (Lang, 1979).

In the period April 1978 to August 1981 some 16 instances of either accidental feeding or lincomycin containing pig feed or the cross-contamination of cattle feed with such medicated pig feed were reported. Analysis of feed samples submitted to the Upjohn Company showed several antibiotics to be present: lincomycin (0-150); penicillin (0-10); tylosin (10-70); dimetridazole (7-9); bacitracin (2-7). (Figures in parentheses are the levels detected, expressed as p.p.m.)

It must be concluded that the consequences of feeding certain antibiotics to non-target species can be severe and that measures to avoid carry-over of

a medicated ration into non-medicated rations (especially those intended for other species) should be strictly observed. Within the UK, a code of practice for feed compounders has been produced and Wilcox (1981) has reviewed some of the corrective actions which can be undertaken in areas of potential cross-contamination of feed.

Consequences to operators

Mill operatives or farmers who routinely add medicaments to feed are potentially exposed to frequent and large amounts of the active ingredient either as inhaled dust or by skin surface exposure. Good manufacturing practices should reduce the potential exposure, especially to drugs which have shown to have mutagenic properties, e.g. carbadox, dimetridazole and ronidazole

Table 33.3 RESISTANCE OF 51 *Staphylococcus aureus* STRAINS FROM 15 BROILER FARMS AND OF 11 *Staphylococcus aureus* STRAINS OBTAINED FROM EMPLOYEES

Drug	Sensitive		Resistant		Sensitive		Resistant	
	No. strains	%	No. strains	%	No. strains	%	No. strains	%
Penicillin	16	31.4	35	68.6	8	72.7	3	27.3
Ampicillin	16	31.4	35	68.6	8	72.7	3	27.3
Oxacillin	51	100.0	0	0	11	100.0	0	0
Cephalothin	51	100.0	0	0	11	100.0	0	0
Tetracycline	22	43.1	29	56.9	8	72.7	3	27.3
Chloramphenicol	49	96.1	2	3.9	11	100.0	0	0
Gentamicin	51	100.0	0	0	11	100.0	0	0
Streptomycin	31	60.8	20	39.2	7	63.6	4	36.4
TMP/SMZ ^a	51	100.0	0	0	11	100.0	0	0
Erythromycin	51	100.0	0	0	11	100.0	0	0
Oleoandomycin	50	98.0	1	2.0	11	100.0	0	0
Tylosin	51	100.0	0	0	11	100.0	0	0
Spiramycin	51	100.0	0	0	11	100.0	0	0
Virginiamycin	51	100.0	0	0	11	100.0	0	0
Lincomycin	51	100.0	0	0	11	100.0	0	0

^a TMP/SMZ = trimethoprim/sulphamethazine.

(Oud, Reutlinger and Branger, 1979). Another possible consequence to the use of antibiotics in feed is the transference to farmworkers of highly resistant microbial populations. In a study conducted on broilers on farms and their employees in Germany, V. Schafer, H. Knothe and W. Lenz (personal communication) have demonstrated that transfer of antibiotic-resistant *Staphylococcus aureus*, from chicken to humans in direct daily contact with the poultry, is not common (Table 33.3).

Overall, the incidence of additive-associated problems with all operatives is very low and compares favourably with other parts of the livestock trade.

Consequences to consumers

This aspect has probably received more attention than any other in terms of both consumer pressure for improved safety levels and scientific attempts to

accurately define the limits of any health risk. Many studies have attempted to correlate the feeding of antibiotics to animals with emergence of antibiotic-resistant organisms in man. However, it is also possible to argue that specific cases of rapidly emerging drug-resistant pathogens are entirely man made, e.g. the spread of antibiotic-resistant gonorrhoea. The actual transference of resistant organisms from animals to man is, however, only one aspect of potential concern to consumers—the residues of active ingredients and their metabolites in the food chain being of equal concern. The sources of antibiotic residues in human food include both therapeutic and growth-promotant use of drugs. In the former category are the range of intramammary products used for the treatment of mastitis in the lactating or dry cows. Much attention has been given to developing a standardized, sensitive test for the presence of antibiotics in milk, with emphasis on the detection of trace levels (0.05 iu) of penicillin. Currently, a commercial test (Delvotest P, Gist-Brocades) exhibits sensitivity to 0.003 iu penicillin and from 0.003 to 10 µg for other agents (Table 33.4).

Table 33.4 SENSITIVITY LIMITS FOR ANTIBIOTICS
AGAINST *Bacillus stearothermophilus* VAR. *calidolactis*
(DELVOTEST P)

<i>Antibiotic</i>	<i>Minimum detectable level</i>
Penicillin	0.003 iu
Cloxacillin	0.02 µg
Nafcillin	0.007 µg
Ampicillin	0.003 µg
Oxytetracycline	0.2 µg
Chloramphenicol	8 µg
Streptomycin	5 µg
Neomycin	2 µg
Kanamycin	10 µg
Erythromycin	1.25 µg
Spiramycin	0.2 µg

The residues of in-feed antibiotics are similarly monitored by companies developing the active ingredients. Each licensed product in the UK carries its own specific recommendation for pre-slaughter withdrawal of the antibiotic and the responsibility for observing the withdrawal time rests first with the prescribing veterinary surgeon and finally with the producer. It is unlikely that much deliberate contravention occurs of the warnings issued by pharmaceutical companies, but as has already been recorded, feedstuffs can contain high levels of medication completely unknown to both veterinarian and farmer.

The final point in this overview attempts to draw a true perspective and concerns the degree to which consumer risk exists. The most recent report of the UK Office of Population Censuses and Surveys analyses causes of death in the country and out of a total of 544 349 deaths in 1980, none was identified as the result of antibiotic residues from feed or as the result of allergies to milk residues. The adverse consequences to UK consumers, in the present era of regulatory pressures on the drug manufacturers, and monitoring of the farmers' observance of antibiotics withholding times, are apparently low.

Interactions with other drugs

It would be a fallacy to believe that most animals receiving medicated feed were not also likely to receive some other medication during their life-span. Many of the widely used additives are fed for virtually the entire production cycle of the species and attention must be paid to possible interactions between the antibiotic in feed and any therapeutic drugs. A recent review of the subject of important veterinary adverse drug interaction highlighted a need for caution when theoretical interactions were predicted or when interactions only relevant to human medicine were proposed (Reilly and Isaacs, 1983). The evidence included here has been summarized to include only those interactions in which antimicrobial agents have a significant role.

A direct inhibitory effect of tetracycline or erythromycin on the action of penicillin has been documented and animals receiving tetracycline medicated feed should therefore not be therapeutically treated with penicillin. Similarly, the effects of tetracycline may be reduced if it is fed in ration containing other additives which contain high levels of calcium, magnesium or aluminium ions, as these can be chelated by the combination. An increased adsorption of lincomycin to kaolin if fed in the same ration will reduce the eventual absorption of the antibiotic, and such a combination should be avoided.

As well as physical interactions, metabolic processes may be altered by one agent, so that the normal metabolism of antibacterial substances is impaired. For example, chloramphenicol can alter liver enzyme pathways so that a decreased metabolism of erythromycin, penicillin, sulphonamides or tetracyclines occurs. This, in turn, could theoretically produce an increased half-life of the active ingredient and could alter eventual tissue residue times.

A last point in respect of interactions concerns the effect of pH on the activity of certain antibiotics. Low pH conditions inhibit the antibacterial effects of erythromycin, neomycin and streptomycin while increasing the activity of nitrofurazone and tetracycline. The reverse effects are also seen if high pH, alkaline, conditions prevail.

Drug interaction has not, however, been recognized or documented as a clinical problem arising from the use of antibiotics in agriculture, even though the investigation of the hundreds of possible chemical combinations has not been started. Current legislation allows certain levels of particular ingredients in a feed ration, without any prescription or formal notification, and anyone who has the responsibility for prescribing another in-feed or other therapeutic antibiotics may be unaware of the 'background' into which the new agent will be introduced.

Prevention of problems

Within the UK, the majority of concentrate rations produced for poultry, swine and dairy cattle are milled in part, or in total, by one of 500 feed mills. The number of potential ingredients required to be mixed into these rations is well over 100, and the variation in protein level, amino acid content, etc., of the ration forces many mills to offer a service which includes 200–300 different finished formulations of their feed. There is, therefore, a large potential for carry-over of one or more ingredients from one ration into another. This

potential danger area led to the production, within the UK feedstuffs industry, of a code of practice which aims to reduce the possible inter-species contamination with active ingredients not prescribed for that particular ration. A review of this code of practice (Wilcox, 1981) has already been referred to.

For the future, one must assume that some approved medicated feed additives will exist and it would be desirable for their use to be as safe as possible. A change in law would be required, so that milling procedures could be adapted to a more programmed nature with medicated feeds. At present, the mixing of prescription-only medicines into feed is only permitted once a prescription has been compiled and forwarded to the mill. Such a system prevents the logical scheduling of all batches of feed containing a particular ingredient and further prevents the follow-on with non-medicated feeds for the same species. The present requirements actually offer the maximum possibilities for both inter-species, cross-contamination and for intra-species medication of allegedly non-medicated rations by requiring mills to intercept mixing schedules with medicated batches of feed as and when prescriptions arrive. Permission to mix and store standard medicated feeds as finished rations against the future issue of a prescription would reduce this risk.

Alternative routes of medication will be developed, e.g. water medication, slow-release formulations, but the farmer assumes that agricultural buildings are plumbed with a capability for water proportioners and the latter still awaits more sophisticated delivery systems to ensure even distribution of the antibiotic.

Conclusions

The actual risk demonstrated to target species, consumer, mill operators and to the activity of other drugs are few, although concern about potential side-effects is rightly a matter for both drug regulatory authorities and for consumer protection bodies. The potential and actual risk to non-target species of in-feed antibiotic medications must be reduced by a combination of improved equipment and drug design, of changes permitting some storage of medicated finished feeds and of responsible observance of the code of practice on cross-contamination of feeds. The benefits of antibiotics in agriculture are discussed elsewhere in this book (see Chapters 12–14, 25 and 29) and it would be foolhardy to lose the benefits for imagined problems. Any restrictive actions should, therefore, be scientifically based and not merely theoretically created.

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INHIBITORY SUBSTANCES IN ANIMAL FEEDS: EXPERIENCES OVER THE PAST FIVE YEARS

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Introduction

An inhibitory substance in an animal feed is regarded as an undeclared additive that can be detected by microbiological assay and may cause deleterious effects to animals. The work discussed refers, in the main, to studies on inhibitory substances in dairy concentrates.

Background

The possibility of cross-contamination of additives in feedstuffs during manufacture has always been a risk, as has the danger that non-target species of animals might become affected by inadvertent exposure to feed additives. For example copper, used in pig food, is toxic to sheep, and monensin, used for beef animals and broiler chickens, is toxic to horses and quail. The Feed Trade Association drew up a voluntary scheme to declare the presence and levels of certain additives included in feeds during manufacture. The Voluntary Additives Scheme operated throughout the 1960s until replaced by the Medicines Act, 1968. In 1976, as a result of concern expressed by the Veterinary Products Committee of the Medicines Commission, a working group was set up to consider the manufacturing practice of medicated animal feedstuffs, and the cross-contamination hazards. The group was particularly concerned with the risk of carryover of medicinal additives from batches of medicated feeds to subsequent mixes of non-medicated feed. A small survey carried out at 12 mills in the south-west of England showed that practices to reduce cross-contamination varied and that despite the precautions some degree of carryover occurred. However, it was concluded that under practical conditions it was impossible to eliminate the cross-contamination completely. As a result, advice was sought concerning the minimum levels of antimicrobial substances which might prove hazardous, and the view was taken that any detectable residues of medicinal products constituted contamination. The United Kingdom Agricultural Supply Trade Association (UKASTA) was asked to draw up a Code of Practice for use by feed manufacturers to minimize the risk of cross-contamination. The code is in two parts. First,

general guidelines of design handling of materials, etc., and secondly, specific instructions for each mill. The code came into effect at the end of 1980. The National Farmers' Union also produced a similar Code of Practice to minimize cross-contamination during on-farm mixing.

Lincomycin cross-contamination

There is little doubt that the contamination of dairy compounds by lincomycin that occurred in 1978/79 highlighted the need for the new Code of Practice. The antibiotic, used in pig feed to control or treat swine dysentery, was granted a product licence in the UK in December 1977, as a prescription-only additive. In late 1978, several cases of sudden drop in milk yield were reported to the ADAS Veterinary Investigation Centres at Gloucester and Langford (near Bristol) and to the ADAS Nutrition Chemistry Department at Bristol. There was a similar history in all cases, namely loss of appetite after the feeding of a new batch of proprietary feed, followed by loss of milk and sometimes acetonaemia or scouring, with an improvement when the compound was replaced by a fresh supply. Milk yields were seriously affected: one farmer reported a 60% reduction in milk yield.

Samples of dairy compounds were submitted to the ADAS Microbiology Department, Bristol, for examination for the presence of moulds and mycotoxins. Samples were also tested for the presence of inhibitory substances. No mycological problems were found in any of the samples, but between November 1978 and March 1979, 10 out of 15 samples examined were found to contain an inhibitory substance. Dairy compounds manufactured by five commercial firms were affected. The inhibitory substances detected were not identified by the Microbiology Department, but reports from private practitioners and contacts at feed firms suggested that batches of dairy compound had been contaminated by lincomycin. Various contacts indicated that the most likely cause was cross-contamination by residues of a previous batch of medicated pig feed, although in other cases it was admitted that pig feed returned from a farm affected by swine vesicular disease had been mistakenly incorporated into dairy compounds. One compounder also suggested that cross-contamination may occur in delivery lorries, and another that one source of contamination had been a mislabelled batch of additive, although this was not confirmed.

Samples of these suspect feeds were submitted to the Microbiology Sub-Division of the Laboratory of the Government Chemist (LGC) London. The LGC receives samples from a variety of sources to confirm the presence of, and to identify, inhibitory substances in feeds. The LGC provides an analytical service to other government departments and is designated as a referee laboratory *inter alia*, when results obtained by official agricultural analysts, who test on behalf of local authorities, are in dispute. The laboratory also participated in the survey of mills already referred to. Samples are examined for the presence and concentration of declared additives under the Medicines Act, or for the presence of deleterious substances under the Agriculture Act. A summary of some recent results is shown in *Table 34.1*.

Methods for the detection and identification of antibiotics occurring as residues in milk and meat and as additives or contaminants in feeds have been

Table 34.1 SUMMARY OF RESULTS OF EXAMINATION OF STATUTORY FEEDSTUFF SAMPLES RECEIVED FROM LOCAL AUTHORITIES

Date	Sample ^a	Antibiotic declared (mg/kg)	Antibiotic(s) found (mg/kg)	Pass/fail
1978	Pig feed	Avoparcin 20	Avoparcin 5.7	F
	Pig pellets	Avoparcin 40	Avoparcin 20 Tetracycline c. 100	F
1979	Broiler starter	Zinc bacitracin 20	None found	F
1980	Poultry starter	Avoparcin 20	Avoparcin 22 Chlortetracycline 5-10	F
	Turkey starter	Zinc bacitracin 20	Zinc bacitracin 6.5	F
	Turkey starter	Zinc bacitracin 30	Zinc bacitracin 2.1	F
	Feed supplement	Tylosin 20 g/kg	Tylosin 6.3 g/kg Penicillin 100	F
1981	Pig feed	Avoparcin 10	Avoparcin 10.7 Tetracycline 10-20	F
	Pig feed*	None	Unidentifiable antibacterial substance	P?
	Dairy feed*	None	None	P
	Pig feed*	None	None	P
1982	Poultry mash	Zinc bacitracin 20	Zinc bacitracin c. 18 ^b	P
	Turkey pellets	Zinc bacitracin 20	Zinc bacitracin c. 7 ^b	F?
	Calf starter	Flavomycin 6	Flavomycin 1	F
	Pullet meal	Zinc bacitracin 10	Zinc bacitracin < 5	F
	Calf starter	Flavomycin 6	Flavomycin 2.4	F

^a All were Medicines Act referee samples, except those indicated by an asterisk which were examined for suspected deleterious substances (Agriculture Act Part IV).

^b Approximate result, assay invalid.

studied. The method of identification and quantification of antibiotics using high-voltage electrophoresis was described by Smither and Vaughan (1978).

Lincomycin at concentrations ranging from 0.05 to 3 mg/kg was found in 9 out of 25 samples of feed tested between September 1978 and September 1979. Other antibiotics detected, sometimes in the same sample as lincomycin, were tylosin, penicillin, chlortetracycline and virginiamycin (Lott and Vaughan, 1983). Similar cases of suspected cross-contamination were reported in the *Veterinary Record* (Anon., 1979; Lang, 1979; Woodger, 1979; Crossman and Poyser, 1981).

Red urine syndrome

In five of the reported cases of drop in milk yield referred to the ADAS departments at Bristol, a red urine syndrome was also described. This was characterized by the production of urine which turned bronze/red on exposure to air. In two cases, feed samples were examined by the LGC and were found to contain tylosin and a mixture of tylosin and penicillin. It was postulated that the antibiotics had altered rumen fermentation, leading to the presence in the urine of skatole which was responsible for the colour (Todd, Lyne and Farmer, 1981). Contacts at feed firms have detailed other instances of red urine syndrome where virginiamycin and tylosin were detected in feed. However, experimental oral administration of 60-240 mg tylosin/head per

day to Friesian dairy cows in the USA did not affect feed intake or milk yield and no symptoms of ill-health were observed (McGuffey, Green and Mackinnon, 1982, unpublished results).

The effects of other milling errors on other groups of animals has been reported: monensin to horses (Stoker, 1975; Matsuoka, 1976; Ordridge, Schubert and Stoker, 1979), monensin to turkeys (Stuart, 1978), monensin to guinea fowl (Kemp, 1978), monensin to fattening cattle which received 10 times the recommended concentration (Collins and McCrea, 1978) and 4-hydroxy-3-nitrophenylarsonic acid (3 nitro) to turkeys (Wise, Hartley and Fowler, 1974).

Inhibitory substances test

The inhibitory substances test used by ADAS microbiologists had been developed at the then NAAS Microbiology Laboratory, Shardlow, Derby, in the 1960s when there had been cases of cross-contamination of penicillin from medicated pig feeds to dairy compounds. In principle, the test involves the extraction of the feed using acetone phosphate buffer at pH 7.5 and a plate assay using *Bacillus stearothermophilus* var. *calidolactis* (*B. cal.*), an organism used to detect antibiotics, particularly benzyl penicillin in milk (Igarashi *et al.*, 1961; Galesloot and Hassing, 1962; Crawford and Galloway, 1964). The test is the method for the detection of penicillin in milk recommended by the International Dairy Federation (IDF, 1970) and was the official first action in the USA (Ouderkerk, 1979). The organism is incubated at 55°C for 3–4 h, at which time zones of inhibition are visible. See Appendix I for details.

The test is simple and cheap to carry out and gives a result within half a working day. However, it is only a qualitative screen for unknown inhibitory substances and it is not possible to identify the inhibitor present. Other microbiological assays using bacteria such as *B. cereus*, *B. subtilis*, *Escherichia coli*, *Micrococcus luteus* and *Streptococcus thermophilus* are also qualitative unless the identity of the inhibitor is known, in which case the test is carried out under the optimum conditions for that inhibitor. The assay methods, for example those of The Medicines (Animal Feedingstuffs) (Enforcement) Regulations 1983 (Anon., 1983), are normally satisfactory, but the presence of antibacterial substances additional to the one being assayed may lead to aberrant results. For example, a feed declared to contain 20 mg/kg avoparcin gave a result of 500 mg/kg when examined by an agricultural analyst. When referred to LGC, it was found to contain undeclared and therefore illegal tetracycline at a concentration between 5 and 10 mg/kg. The substitution of a tetracycline-resistant bacterial culture for the assay organism prescribed in the official method enabled a correct result to be obtained for avoparcin of 22 mg/kg, well within the permitted tolerance for addition.

As a control, when using the inhibitory substances test, penicillinase has been included to check if inhibition was due to penicillin. However, recent studies have indicated that the form of penicillinase used is not pure and that reduction of inhibition is not, in fact, reliable as confirmation of the presence of penicillin as the inhibitor.

The detection of lincomycin by the screening method employed may be considered fortuitous in that the antibiotic was soluble in acetone phosphate

buffer and that *B.cal* was very sensitive to the antibiotic. These findings indicated the need to study the effect of other additives and some mycotoxins on the test and led to further collaborative work between the ADAS Microbiology Discipline and the LGC.

EFFECT OF FEED ADDITIVES AND MYCOTOXINS ON INHIBITORY SUBSTANCES TEST

Tests were carried out at the Microbiology Department, Bristol, using *B. cal* in plastic assay plates (Nunc) with 36 wells in a 6 × 6 Latin Square using 5 dilutions of additives plus controls. Solutions of additives were prepared in acetone phosphate buffer to give 10 ×, 5 ×, 1 ×, 0.5 × and 0.1 × recommended inclusion rates, except for lincomycin which was used at 0.2–0.002 × its recommended rate of inclusion in pig feed. Sixty microlitres of test solution

Table 34.2 ESTIMATED MINIMUM CONCENTRATIONS OF FEED ADDITIVES DETECTABLE BY INHIBITORY SUBSTANCES TEST

Additive	Active ingredient(s)	Estimated minimum detectable concentration of active ingredients (mg/kg)	Detectability (%) ^a
Avotan 50	Avoparcin	2.0	5.0
Payzone 250	Nitrovin	1.0	4.0
Eskalin 500	Virginiamycin	0.7	3.5
Zinc bacitracin	Zinc bacitracin	3.0	3.0
Lincocin	Lincomycin-HCl	0.1	1.0
Elancoban	Monensin sodium	1.0	0.8
Tylasul	{ Tylosin Sulphadimidine }	{ 0.02 0.02 }	0.2
Tylan	Tylosin	0.01	0.025

^a Estimated minimum detectable concentration as a percentage of maximum recommended inclusion rate.

was added to each well. Plates were incubated at 55°C for 3 h. After incubation, zone sizes were measured and graphs were prepared of mean zone size against log concentration of additive. The minimum detectable concentration was calculated by extrapolation. The most potent antibiotics tested in terms of the inhibitory test, were tylosin, monensin and lincomycin (*Table 34.2*). Other additives shown to be inhibitory, although no minimum detectable concentration could be calculated, are shown in *Table 34.3*, whereas *Table 34.4* lists other additives not inhibitory to the test at normal rates of inclusion.

Table 34.3 OTHER ADDITIVES SHOWN TO BE INHIBITORY—MINIMUM DETECTABLE CONCENTRATION NOT DETERMINED

Additive	Active ingredient(s)	Concentration of active ingredient in finished feed (mg/kg)
Aurofac 100	Chlortetracycline hydrochloride	200–600
Copper sulphate	Copper	50–200
Cyfac	{ Chlortetracycline hydrochloride Procaine penicillin Sulphadimidine }	{ 165 82 165 }
Nifulidone	Furazolidone	230–460

Table 34.4 ADDITIVES NOT INHIBITORY TO INHIBITORY SUBSTANCES TEST AT NORMAL RATES OF INCLUSION IN FEEDS

<i>Additive</i>	<i>Active ingredient(s)</i>	<i>Concentration of active ingredient in finished feed (mg/kg)</i>
Emtryl	Dimetridazole	75-200
Fortigros	{ Carbadox	50
	{ Sulphadimidine	100
3-Nitro	4-Hydroxy-3-nitrophenylarsonic acid	25-50
Quixalud	Halquinol	120-600
Salcostat	3,5-Dinitro- <i>o</i> -toluamide	62.5-125
Sulfuride	Nifursol	50
Sulphamethazine	Sulphadimidine	100

Eight mycotoxins were also tested using two dilutions of each. Mycotoxins at the concentrations likely to occur in feed do not affect the test. The results are shown in *Table 34.5*.

Table 34.5 MYCOTOXINS NOT INHIBITORY TO INHIBITORY SUBSTANCES TEST

<i>Mycotoxin</i>	<i>Concentration in feed (mg/kg)</i>
Aflatoxin B ₁	1 800
Citrinin	1 800
Diacetoxyscirpenol	20 000
Ochratoxin A	1 800
Patulin	1 000
Sterigmatocystin	1 000
T ₂ toxin	20 000
Zearalenone	900

NON-SPECIFIC INHIBITORS

Until 1979, samples of control dairy compounds not suspected of containing inhibitors had consistently given negative results to the *B. cal* test. However, during that year, random tests on compound feeds not associated with any specific problem did show inhibition to *B. cal*. This organism is known to be more sensitive than other assay microorganisms to some antibiotics and other inhibitors, so that samples showing inhibition to *B. cal* were also checked against *B. cereus* and *M. luteus*. In most cases no, or very slight, inhibition was found with the other microorganisms. Samples were referred to the LGC and in most cases any inhibition was described as non-specific. The effect of these inhibitors is very similar to naturally occurring inhibitors found in meat, which are thought to be long-chain fatty acids or derivatives (Smither, 1978).

The finding of non-specific inhibitors in animal feeds is a fairly recent phenomenon and may be associated with the inclusion of different types of fats in dairy compounds. It is known that different fats affect rumen fermentation in different ways and that the sudden inclusion of unusual fat in a diet

may upset the rumen sufficiently to cause some effect in milk yield. Further collaborative work is being carried out on the presence of these non-specific inhibitors.

Discussion

Many of the reported cases of inhibitory substances in feed have been very difficult to follow up because of a natural reticence of feed firms to allow investigation by outside bodies. It is also recognized that farmers often blame any problems with stock onto a new batch of feed. It has been known for cows initially to reject a new batch of feed because of the presence of different spices or flavourings. All investigations are retrospective and the feed has often been replaced on the farm, or wrong samples were submitted. Also, it is likely that contamination by an antibiotic will not be uniform and it may be that only the first part of a batch through the mill will be affected.

The reporting to farmers, by ADAS microbiologists, of the presence of inhibitory substances has been criticized by some feed companies on the grounds that no identification of the inhibitor has been made and that there is normally antibacterial activity in dairy compounds. This latter statement was not verifiable until 1979. This criticism has led ADAS microbiologists to reduce the number of tests being carried out on samples. However, if the future collaborative work with LGC is successful, it may be possible to continue tests on a different basis.

It had been hoped that the new UKASTA Codes of Practice would largely eliminate cases of cross-contamination to dairy compounds; however, further cases of a sudden drop in milk yield have recently been investigated which resulted in immediate replacement of the suspect feed and compensation for loss of milk yield by the feed firm concerned.

Appendix I: Examination of animal feedstuffs for inhibitory substances

PRINCIPLE

The method is adapted from the test described by Galesloot and Hassing (1962) to detect antibiotics and inhibitory substances in milk. The test micro-organism is *Bacillus stearothermophilus* var. *calidolactis*. The inhibitory substances are extracted from the feedstuff using acetone phosphate buffer. Seeded plates of the organism are prepared and inhibition demonstrated by the use of either filter paper discs soaked in extract placed on the surface of the agar or filling wells cut in the agar. Zones of inhibition can be seen after incubation at 55°C for 3–4 h.

MATERIALS

- (1) *Test organism: Bacillus stearothermophilus* var. *calidolactis*, Strain C953 (Netherlands Institute for Dairy Research), obtained from National Institute for Research in Dairying, Shinfield, Reading, catalogue no. 1780.

- (2) *Slant agar*: Oxoid CM3 Nutrient Agar, reconstituted and sterilized as directed and prepared as slants in bijou bottles.
- (3) *Tryptone dextrose yeast extract broth (TDYEB)*

Yeast extract	10 g
Tryptone	20 g
Glucose	0.5 g
Distilled water	1000 ml

Dispensed in 10 ml quantities in 100 ml bottles. Sterilized at 121°C for 15 min. Final pH, 8.0 ± 0.1 .

- (4) *Plate count agar*: Oxoid CM325 PCA, reconstituted and sterilized as directed. Dispensed in 50 ml quantities in 100 ml bottles. Final pH, 8.0 ± 0.1 .
- (5) *Acetone phosphate buffer*

Disodium hydrogen phosphate (Na_2HPO_4)	9.78 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.85 g
Distilled water (hot)	200 ml
Acetone	250 ml
Distilled water	to 1000 ml

Dissolve salts in 200 ml hot distilled water and cool. Add 250 ml acetone and make up to 1000 ml with distilled water. Final pH, 7.5.

- (6) *Filter discs/wells*; Whatman Discs AA, 6 mm diameter, or flame-sterilized 6 mm diameter cork borer.
- (7) *Standard penicillin solution*. Stock solution containing 100 iu/ml prepared by dissolving sodium or potassium benzyl penicillin in sterile distilled water and stored at 5°C for up to 1 month. Control solutions prepared on the day of testing by preparing a solution containing 1 iu/ml in distilled water and then solutions containing 0.01 and 0.02 iu/ml in acetone phosphate buffer.

METHOD

- (1) *Preparation of test culture*. The culture was maintained on a nutrient agar slant at 5°C. Before use, the organism was subcultured onto a fresh slant and incubated at 55°C for 48 h. TDYEB, 10 ml, was inoculated with a loopful of culture from the slant and incubated, with the bottle laid on its side to allow maximum surface area, at 55°C for 18 h.
- (2) *Preparation of test plates*. Sterile PCA, 50 ml, was melted and cooled to 55°C. Freshly prepared test culture, 10 ml, was added and mixed thoroughly. Sufficient agar to give a layer of medium 0.8–1.0 mm deep was poured into petri dishes or assay plate (Nunc), previously warmed to 55°C, and allowed to solidify on a cool horizontal surface. If not required immediately, the plates were stored in sealed polythene bags at 5°C.
- (3) *Preparation of extract*. Feed, 50 g, was added to 200 ml acetone phosphate buffer, shaken and allowed to stand for 2 h at room temperature. It was occasionally necessary to grind some samples before extraction.
- (4) *Test method*. Plates were marked on the underside for identification of

sample and controls. Using sterile forceps, an assay disc was dipped into the extract or control solution, excess liquid was removed by touching onto the side of the container, and the disc placed on the agar and pressed down gently with forceps. Each sample was tested at least in triplicate. The forceps were rinsed in sterile distilled water between samples. Alternatively, 6 mm wells were cut in the agar using a flame-sterilized cork borer and the wells filled with 60 µl of extract or control. The plates were incubated at 55°C for 3–4 h and observed for zones of inhibition.

INTERPRETATION

Clear zones of inhibition around the extract discs/wells indicate the presence of an inhibitory substance in the feed under examination. The size of zone gives an indication of the potency of the inhibitor against the organism. Zone diameter around the penicillin control disc should be approximately 10 mm for 0.01 iu/ml and 15 mm for 0.02 iu/ml. Absence of clear zones of inhibition may be due to error in technique, loss of potency of penicillin solution or insensitivity of the culture due to mutation. In such circumstances, the tests were repeated using a fresh culture and newly prepared penicillin solutions.

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THE EFFECTS OF NISIN ON THE SENSITIVITY OF MICROORGANISMS TO ANTIBIOTICS AND OTHER CHEMOTHERAPEUTIC AGENTS

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Introduction

Following early observations by Rogers and Whittier (1928) and Whitehead and Riddet (1933) of the inhibitory activity of certain strains of *Streptococcus lactis* on lactic fermentation in yoghurt and cheese manufacture, the metabolite responsible was isolated and partially characterized by Whitehead (1933) and Cox and Whitehead (1936). It was studied in greater detail by Mattick and Hirsch (1947), who gave the name nisin to the polypeptide antibiotic produced by Lancefield Group N strains of *S. lactis*.

The antibiotic found a major application in the food industry, where its activity as a preservative has been widely studied (O'Brien *et al.*, 1956; Hawley, 1958; Campbell, Sniff and O'Brien, 1959; Heinemann, Voris and Stumbo, 1965); its preservative activity was shown by Thorpe (1960) to be due not to the sensitization of spores to heat treatment, but to the prevention of outgrowth of spores which survived heat-treatment processes, including the mesophilic anaerobes in cheese spreads and the thermophilic aerobes and anaerobes in low acid canned foods (Lipinska, 1977; Fowler, 1979).

In view of the antibacterial activity of nisin against a number of Gram-positive organisms (staphylococci, streptococci, corynebacteria and *Mycobacterium tuberculosis*), some interest was shown by Hirsch and Mattick (1949) in its use as a therapeutic antibiotic, but it has never been used clinically.

Nisin is inactivated by a 'nisinase' produced by some bacteria (Alifax and Chevalier, 1962; Jarvis, 1967), but studies with strains of *Micrococcus pyogenes* var. *aureus* by Szybalski (1953) showed no evidence of cross-resistance with other antibiotics. Resistance to nisin, and cross-resistance with other antibiotics, is in any case unlikely to develop *in vivo*, since nisin has been shown by Cowell, Allen and Jarvis (1971) to have no effect on the microflora of the oral cavity, and by Jarvis and Mahoney (1969) to be rapidly destroyed by α -chymotrypsin digestion.

However, when present in foods nisin may come into contact, at sub-lethal concentrations, with a variety of organisms of importance in terms of food

hygiene and enteric infections. That this might induce the development of strains resistant to nisin and cross-resistant with commonly used therapeutic antibiotics is of obvious public health concern, and the object of this study was to re-evaluate the effects of nisin in relation to a wide range of currently used therapeutic antimicrobial substances.

Materials and methods

CULTURES

Five strains each of *Escherichia coli* and *Staphylococcus aureus*, one strain each of *Shigella flexneri*, *Sh. dysenteriae*, *Sh. sonnei* and *Sh. boydii* and one strain each of *Salmonella virchow*, *S. dublin*, *S. anatum*, *S. enteritidis* and *S. typhimurium* were used. The majority were obtained originally from veterinary sources, but within each genus at least one of the strains was obtained from the National Collection of Type Cultures.

The organisms were maintained in a liquid nitrogen cryostat. For use as inocula, they were grown in tryptone soy broth at 37°C for 18 h and the cultures diluted with sterile saline to give a viable count of between 5×10^5 and 5×10^6 cfu/ml.

CHEMOTHERAPEUTIC ANTIBIOTICS

The antibiotics (in addition to nisin) used in the cross-resistance study were ampicillin, bacitracin, benzylpenicillin, carbenicillin, chloramphenicol, erythromycin, gentamicin, lincomycin, nalidixic acid, neomycin, nitrofurantoin novobiocin, oxtetracycline, polymyxin B, rifampicin, streptomycin and sulphamerazine.

MINIMUM INHIBITORY CONCENTRATION DETERMINATIONS

Serial two-fold dilutions of each of the antibiotics were prepared in 15 ml volumes of Sensitest Agar (Oxoid) to give final concentrations of 1000 to 0.01 µg/ml in the plates. A growth control plate containing no antibiotic was included in each series.

The plates were inoculated with the test organisms using a Denley Multi-point Inoculator adjusted to deliver 1 µl of each inoculum. The plates were incubated aerobically at 37°C for 24 h and the minimum inhibitory concentrations recorded.

PASSAGE

Each of the organisms was serially sub-cultured daily for 10 days in tryptone soy broth containing a sub-inhibitory concentration of nisin incubated at 37°C for 24 h. A control series of sub-cultures in broth containing no nisin was also prepared.

The concentrations of nisin used were 500 µg/ml for the *Salmonella* spp., *Shigella* spp. and the strains of *E. coli* and 2.5 µg/ml for strains of *Staphylo-*

coccus aureus. The current addition levels of nisin to foods range between 2.5 µg/g and 12.5 µg/g.

The cultures which had been passaged in nisin and their corresponding control cultures were then used to prepare inocula for determination of their sensitivities to each of the antibiotics. These determinations were carried out in duplicate.

Results

The minimum inhibitory concentrations (MICs) obtained with nisin for each of the test organisms before and after passage are shown in *Table 35.1*, from which it can be seen that all of the Gram-negative bacteria were insensitive to nisin with MICs of greater than 1000 µg/ml, that the staphylococcal strains were initially sensitive with MICs of 8–16 µg/ml and that, following passage in nisin, they became less sensitive with MICs in the range of 125–500 µg/ml.

The volume of data generated in testing the 17 antibiotics against two sub-strains of 19 test organisms is presented in *Tables 35.1–35.9*. In interpreting the results of cross-resistance studies with the 17 chemotherapeutic antibiotics, the criterion adopted as indicative of a significant change in sensitivity was a greater than two tube-dilution difference between the MICs obtained with the strain passaged in nisin and its corresponding control culture. Apart from the results obtained with nisin itself, none of the results obtained showed more than a two tube-dilution difference between an inoculum passaged in the presence of nisin and its counterpart passaged in medium alone.

Table 35.1 MICs OF NISIN AND CARBENICILLIN FOR ORGANISMS PASSAGED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Nisin		Carbenicillin	
	Nisin-free medium	Passage in Nisin-containing medium	Nisin-free medium	Passage in Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	16	125	4	4
<i>S. aureus</i> HRC 134	16	500	0.5	0.5
<i>S. aureus</i> HRC 135	16	250	0.5	1
<i>S. aureus</i> HRC 136	16	250	2	4
<i>S. aureus</i> NCTC 6571	16	250	1	1
<i>Escherichia coli</i> HRC 46	> 1000	> 1000	8	16
<i>E. coli</i> HRC 47	> 1000	> 1000	32	32
<i>E. coli</i> HRC 49	> 1000	> 1000	8	32
<i>E. coli</i> HRC 51	> 1000	> 1000	8	32
<i>E. coli</i> NCTC 8739	> 1000	> 1000	8	4
<i>Shigella flexneri</i> HRC 67	1000	> 1000	1	2
<i>Sh. dysenteriae</i> NCTC 2966	1000	> 1000	1	4
<i>Sh. sonnei</i> HRC 66	> 1000	> 1000	64	125
<i>Sh. boydii</i> NCTC 9328	> 1000	> 1000	2	2
<i>Salmonella virchow</i> HRC 41	> 1000	> 1000	8	8
<i>Sal. dublin</i> HRC 36	> 1000	> 1000	2	8
<i>Sal. anatum</i> HRC 26	> 1000	> 1000	8	8
<i>Sal. typhimurium</i> HRC 1	> 1000	> 1000	8	8
<i>Sal. enteritidis</i> NCTC 6676	> 1000	> 1000	16	8

Table 35.2 MICs OF AMPICILLIN AND BACITRACIN FOR ORGANISMS PASSAGED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Ampicillin		Bacitracin	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	4	2	125	250
<i>S. aureus</i> HRC 134	0.25	0.25	250	250
<i>S. aureus</i> HRC 135	0.5	0.25	250	250
<i>S. aureus</i> HRC 136	1	2	250	500
<i>S. aureus</i> NCTC 6571	0.5	0.5	16	32
<i>Escherichia coli</i> HRC 46	8	16	1000	1000
<i>E. coli</i> HRC 47	8	16	> 1000	1000
<i>E. coli</i> HRC 49	8	16	> 1000	> 1000
<i>E. coli</i> HRC 51	8	8	1000	1000
<i>E. coli</i> NCTC 8739	4	4	> 1000	> 1000
<i>Shigella flexneri</i> HRC 67	4	4	1000	1000
<i>Sh. dysenteriae</i> NCTC 2966	2	4	> 1000	> 1000
<i>Sh. sonnei</i> HRC 66	500	500	> 1000	> 1000
<i>Sh. boydii</i> NCTC 9328	8	8	500	1000
<i>Salmonella virchow</i> HRC 41	4	4	> 1000	> 1000
<i>Sal. dublin</i> HRC 36	4	4	> 1000	> 1000
<i>Sal. anatum</i> HRC 26	4	4	> 1000	> 1000
<i>Sal. typhimurium</i> HRC 1	4	4	> 1000	> 1000
<i>Sal. enteritidis</i> NCTC 6676	4	4	> 1000	> 1000

Table 35.3 MICs OF BENZYL PENICILLIN AND CHLORAMPHENICOL FOR ORGANISMS PASSAGED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Benzyl penicillin		Chloramphenicol	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	0.5	0.25	64	64
<i>S. aureus</i> HRC 134	0.03	0.03	64	64
<i>S. aureus</i> HRC 135	0.03	0.03	125	64
<i>S. aureus</i> HRC 136	0.25	0.5	1000	1000
<i>S. aureus</i> NCTC 6571	0.06	0.06	64	64
<i>Escherichia coli</i> HRC 46	32	64	250	250
<i>E. coli</i> HRC 47	32	64	125	250
<i>E. coli</i> HRC 49	32	64	125	250
<i>E. coli</i> HRC 51	32	32	125	125
<i>E. coli</i> NCTC 8739	8	16	64	125
<i>Shigella flexneri</i> HRC 67	16	32	64	64
<i>Sh. dysenteriae</i> NCTC 2966	8	16	32	125
<i>Sh. sonnei</i> HRC 66	> 1000	> 1000	64	64
<i>Sh. boydii</i> NCTC 9328	32	32	32	32
<i>Salmonella virchow</i> HRC 41	16	8	125	125
<i>Sal. dublin</i> HRC 36	8	8	64	64
<i>Sal. anatum</i> HRC 26	16	8	125	125
<i>Sal. typhimurium</i> HRC 1	16	16	125	125
<i>Sal. enteritidis</i> NCTC 6676	16	16	125	125

Table 35.4 MICs OF ERYTHROMYCIN AND GENTAMICIN FOR ORGANISMS PASSAGED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Erythromycin		Gentamicin	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	1	0.5	4	8
<i>S. aureus</i> HRC 134	1	0.5	2	4
<i>S. aureus</i> HRC 135	1	1	4	4
<i>S. aureus</i> HRC 136	>1000	>1000	2	4
<i>S. aureus</i> NCTC 6571	0.5	0.5	4	8
<i>Escherichia coli</i> HRC 46	125	250	1	1
<i>E. coli</i> HRC 47	125	125	1	1
<i>E. coli</i> HRC 49	250	250	1	1
<i>E. coli</i> HRC 51	64	125	1	2
<i>E. coli</i> NCTC 8739	64	64	0.5	1
<i>Shigella flexneri</i> HRC 67	64	64	1	1
<i>Sh. dysenteriae</i> NCTC 2966	64	125	16	8
<i>Sh. sonnei</i> HRC 66	64	64	16	16
<i>Sh. boydii</i> NCTC 9328	64	64	2	2
<i>Salmonella virchow</i> HRC 41	250	250	0.25	1
<i>Sal. dublin</i> HRC 36	250	250	1	0.5
<i>Sal. anatum</i> HRC 26	250	250	0.5	0.5
<i>Sal. typhimurium</i> HRC 1	250	250	0.12	0.25
<i>Sal. enteritidis</i> NCTC 6676	250	250	0.5	0.5

Table 35.5 MICs OF NALADIXIC ACID AND LINCOMYCIN FOR ORGANISMS PASSAGED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Naladixic acid		Lincomycin	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	64	64	2	2
<i>S. aureus</i> HRC 134	125	125	2	2
<i>S. aureus</i> HRC 135	125	125	2	2
<i>S. aureus</i> HRC 136	250	250	2	1
<i>S. aureus</i> NCTC 6571	64	64	1	1
<i>Escherichia coli</i> HRC 46	8	8	>1000	>1000
<i>E. coli</i> HRC 47	4	4	1000	>1000
<i>E. coli</i> HRC 49	16	8	1000	>1000
<i>E. coli</i> HRC 51	8	4	>1000	>1000
<i>E. coli</i> NCTC 8739	4	4	>1000	>1000
<i>Shigella flexneri</i> HRC 67	4	4	250	500
<i>Sh. dysenteriae</i> NCTC 2966	2	2	500	500
<i>Sh. sonnei</i> HRC 66	4	4	1000	>1000
<i>Sh. boydii</i> NCTC 9328	4	4	250	500
<i>Salmonella virchow</i> HRC 41	8	8	>1000	>1000
<i>Sal. dublin</i> HRC 36	8	4	>1000	1000
<i>Sal. anatum</i> HRC 26	8	8	>1000	>1000
<i>Sal. typhimurium</i> HRC 1	8	8	>1000	>1000
<i>Sal. enteritidis</i> NCTC 6676	8	8	1000	1000

Table 35.6 MICs OF NITROFURANTOIN AND NEOMYCIN FOR ORGANISMS PASSAGED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Nitrofurantoin		Neomycin	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	32	16	2	2
<i>S. aureus</i> HRC 134	32	16	1	2
<i>S. aureus</i> HRC 135	32	32	0.5	2
<i>S. aureus</i> HRC 136	16	16	250	1000
<i>S. aureus</i> NCTC 6571	8	16	2	2
<i>Escherichia coli</i> HRC 46	125	125	4	4
<i>E. coli</i> HRC 47	32	32	8	4
<i>E. coli</i> HRC 49	32	32	4	4
<i>E. coli</i> HRC 51	16	16	4	4
<i>E. coli</i> NCTC 8739	8	16	2	4
<i>Shigella flexneri</i> HRC 67	32	32	8	8
<i>Sh. dysenteriae</i> NCTC 2966	8	8	4	4
<i>Sh. sonnei</i> HRC 66	8	8	4	4
<i>Sh. boydii</i> NCTC 9328	4	4	4	8
<i>Salmonella virchow</i> HRC 41	125	64	2	2
<i>Sal. dublin</i> HRC 36	32	32	1	2
<i>Sal. anatum</i> HRC 26	32	32	2	4
<i>Sal. typhimurium</i> HRC 1	32	32	2	2
<i>Sal. enteritidis</i> NCTC 6676	32	32	2	2

Table 35.7 MICs OF NOVOBIOCIN AND OXYTETRACYCLINE FOR ORGANISMS PASSAGED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Novobiocin		Oxytetracycline	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	0.25	0.25	250	250
<i>S. aureus</i> HRC 134	0.25	0.25	8	8
<i>S. aureus</i> HRC 135	0.25	0.25	8	8
<i>S. aureus</i> HRC 136	0.25	0.25	250	250
<i>S. aureus</i> NCTC 6571	0.5	0.5	4	8
<i>Escherichia coli</i> HRC 46	250	64	1000	1000
<i>E. coli</i> HRC 47	500	250	4	8
<i>E. coli</i> HRC 49	1000	1000	8	16
<i>E. coli</i> HRC 51	250	250	8	8
<i>E. coli</i> NCTC 8739	250	250	8	8
<i>Shigella flexneri</i> HRC 67	64	64	4	4
<i>Sh. dysenteriae</i> NCTC 2966	64	125	16	16
<i>Sh. sonnei</i> HRC 66	500	500	16	16
<i>Sh. boydii</i> NCTC 9328	64	64	2	4
<i>Salmonella virchow</i> HRC 41	1000	1000	8	8
<i>Sal. dublin</i> HRC 36	64	64	4	4
<i>Sal. anatum</i> HRC 26	500	1000	8	8
<i>Sal. typhimurium</i> HRC 1	1000	500	8	8
<i>Sal. enteritidis</i> NCTC 6676	> 1000	> 1000	4	8

Table 35.8 MICs OF POLYMYXIN B AND RIFAMPICIN FOR ORGANISMS PASSED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Polymyxin B		Rifampicin	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	125	250	0.06	0.12
<i>S. aureus</i> HRC 134	125	125	0.03	0.03
<i>S. aureus</i> HRC 135	125	250	0.03	0.06
<i>S. aureus</i> HRC 136	125	125	0.06	0.06
<i>S. aureus</i> NCTC 6571	125	250	0.03	0.03
<i>Escherichia coli</i> HRC 46	1	1	16	16
<i>E. coli</i> HRC 47	1	1	32	32
<i>E. coli</i> HRC 49	2	1	16	32
<i>E. coli</i> HRC 51	1	1	32	32
<i>E. coli</i> NCTC 8739	1	1	16	16
<i>Shigella flexneri</i> HRC 67	0.5	0.5	16	16
<i>Sh. dysenteriae</i> NCTC 2966	0.5	0.5	32	64
<i>Sh. sonnei</i> HRC 66	1	0.5	32	64
<i>Sh. boydii</i> NCTC 9328	0.5	0.5	16	16
<i>Salmonella virchow</i> HRC 41	8	4	16	16
<i>Sal. dublin</i> HRC 36	32	4	16	16
<i>Sal. anatum</i> HRC 26	8	8	32	16
<i>Sal. typhimurium</i> HRC 1	4	16	32	32
<i>Sal. enteritidis</i> NCTC 6676	2	2	32	32

Table 35.9 MICs OF STREPTOMYCIN AND SULPHAMERAZINE FOR ORGANISMS PASSED IN NISIN-FREE AND NISIN-CONTAINING MEDIUM

Organisms	Minimum inhibitory concentration (µg/ml) of:			
	Streptomycin		Sulphamerazine	
	Passage in		Passage in	
	Nisin-free medium	Nisin-containing medium	Nisin-free medium	Nisin-containing medium
<i>Staphylococcus aureus</i> HRC 133	16	16	> 1000	> 1000
<i>S. aureus</i> HRC 134	4	8	> 1000	> 1000
<i>S. aureus</i> HRC 135	4	8	> 1000	> 1000
<i>S. aureus</i> HRC 136	> 1000	> 1000	> 1000	> 1000
<i>S. aureus</i> NCTC 6571	8	8	> 1000	> 1000
<i>Escherichia coli</i> HRC 46	8	8	> 1000	> 1000
<i>E. coli</i> HRC 47	16	16	> 1000	> 1000
<i>E. coli</i> HRC 49	8	16	> 1000	> 1000
<i>E. coli</i> HRC 51	8	16	> 1000	> 1000
<i>E. coli</i> NCTC 8739	8	8	> 1000	> 1000
<i>Shigella flexneri</i> HRC 67	16	16	> 1000	> 1000
<i>Sh. dysenteriae</i> NCTC 2966	8	4	500	> 1000
<i>Sh. sonnei</i> HRC 66	8	8	> 1000	> 1000
<i>Sh. boydii</i> NCTC 9328	8	16	32	32
<i>Salmonella virchow</i> HRC 41	32	32	> 1000	> 1000
<i>Sal. dublin</i> HRC 36	32	32	> 1000	> 1000
<i>Sal. anatum</i> HRC 26	32	32	> 1000	> 1000
<i>Sal. typhimurium</i> HRC 1	32	32	> 1000	> 1000
<i>Sal. enteritidis</i> NCTC 6676	8	8	> 1000	> 1000

Discussion

It was clear from the results obtained that nisin was inactive against the Gram-negative organisms used in this study (*Shigella* spp., *Salmonella* spp. and *Escherichia coli*), since none of the organisms were inhibited by a concentration of 1000 µg/ml, but nisin was active against all five of the staphylococcal strains used, at a concentration of 16 µg/ml.

When the staphylococcal strains were exposed to sub-inhibitory concentrations of nisin by 10 serial passages in a medium containing 2.5 µg/ml, the strains became more resistant to nisin, with four giving MICs in the range of 250–1000 µg/ml, and the other strain in the range 32–500 µg/ml.

In interpreting the results, the criterion adopted as indicative of an experimentally significant change in sensitivity was a two-tube dilution difference in the MIC obtained with the strain passaged in medium alone and in medium containing nisin.

A certain amount of variability was found in the first series of results, both with regard to tests with nisin itself and in the tests using other antibiotics and chemotherapeutic agents designed to determine whether any cross-resistance had developed following passage in sub-inhibitory concentrations of nisin. In this series, if the two tube-dilution difference between results was taken as experimentally significant, then a decrease in sensitivity was seen in 20 out of the 360 determinations, but these were randomly spread between the 17 different chemotherapeutic agents used. It is known that MIC determinations are subject to some degree of variability, but the sets of results obtained in the first series frequently appeared to be showing greater than the expected variability, both in comparison of pre-passage with post-passage results and (assuming that no true cross-resistance had developed) between the two sets of post-passage results. It was due to this apparent variability that the second series of tests was carried out. On this occasion it was decided to omit the pre-passage tests as contributing little to assessment of the results with regard to the development of cross-resistance, and to carry out all of the post-passage determinations on two separate occasions.

The results obtained in the second series appeared to be more mutually consistent than did the results of the first series of tests. The duplicated determinations were rarely more than one tube-dilution apart, and the results of the second series of tests did much to confirm that many of the apparently erratic results obtained in the first series of tests were simply due to normal variation and that most of the results which showed an apparently experimentally significant difference in MIC were due to this variability.

Apart from the results obtained with nisin itself, none of the results obtained showed more than a two tube-dilution difference between an inoculum passaged in medium alone and its counterpart passaged in medium containing nisin itself.

Conclusion

On the basis of the results obtained in this study, it was possible to conclude that exposure of nisin-sensitive organisms to sub-inhibitory concentrations of the antibiotic was capable of inducing increased resistance to nisin itself,

but that organisms which had developed this resistance did not show increased resistance to any of a wide range of antibiotics extensively used in chemotherapy.

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FOOD ANTIBIOTIC NISIN: COMPARATIVE EFFECTS ON *ERYSIPELOTHRIX* AND *LISTERIA*

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Introduction

Nisin is a polypeptide antibiotic produced by *Streptococcus lactis* "N". Since it is non-toxic (Frazer, Sharratt and Hickman, 1962) and not in medical use (FAO/WHO, 1969), it has found applications in food preservation. Rayman, Aris and Hurst (1981) discussed the possible use of nisin as an alternative, or adjunct, to nitrite in meat preservation. Both *Erysipelothrix rhusiopathiae* and *Listeria monocytogenes* are pathogenic to man and have been reported from meat in abattoirs on many occasions. Animal products of diseased animals, or food contaminated during processing or delivery, constitute a potential threat to man. The present work was carried out to determine whether nisin had a comparative inhibitory effect on *E. rhusiopathiae* and *L. monocytogenes* and to investigate other factors which may have an influence on the effectiveness of the antibiotic against these organisms. These included the effect of temperature (22°C or 37°C), pH of the culture media and inoculum size, together with the effect of physiological age of the inoculum (logarithmic and stationary phases).

Materials and methods

ORGANISMS AND MEDIUM

The organisms used were *L. monocytogenes* strain 4379 (highly haemolytic, serovar 5) and strain 10357 (low haemolytic, serovar 1a) and *E. rhusiopathiae* 5380 (serovar 5).

Throughout the experiments the organism were propagated in Nutrient Broth Oxoid No. 2. The pH was checked before and after sterilization and adjusted if necessary.

The stock solution of nisin, growth studies and viable count method were as reported earlier (Mohamed, Seaman and Woodbine, 1981).

Residues of nisin at different pHs were examined by the plate diffusion method of Tramer and Fowler (1964). Haemolysin titration followed the method of Girard and Sbarra (1963).

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Results

Figure 36.1 illustrates the effect of nisin on the growth of *E. rhusiopathiae* at 37°C and pH 7.4. It is evident that increasing nisin concentration in this culture medium from 2 to 8 iu/ml caused increasing reduction in the initial viable number inoculated, with regrowth of the remaining cells, until the antibiotic reached a level (16 iu/ml) where it became completely inhibitory. 8 iu/ml of nisin resulted in a progressive decline of the viable cells until between 8 and 12 h no organisms could be detected (<10 cfu/ml), but after 16 h, regrowth of the few remaining cells started with an apparently similar

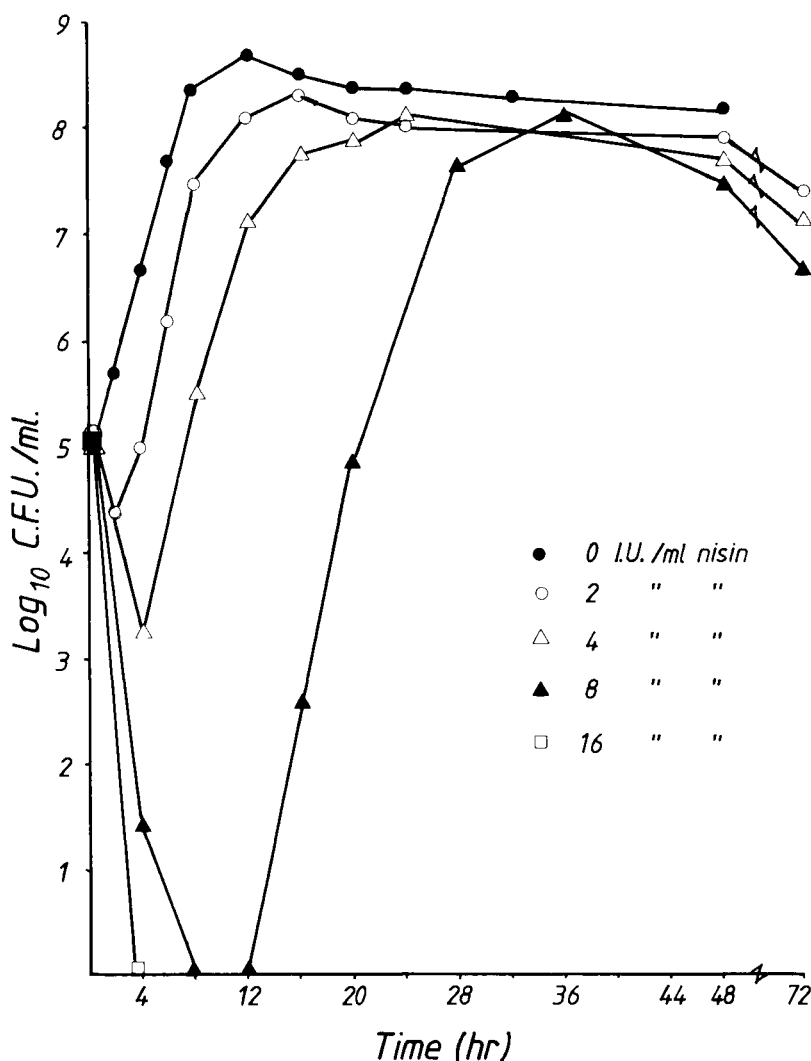


Figure 36.1 Effect of nisin on the growth of *Erysipelothrix rhusiopathiae* (5380) at pH 7.4 and 37°C

growth rate to that of the control, attaining an unaffected maximum viable number.

When *L. monocytogenes* (4379) was examined under the same conditions, with 37°C as incubation temperature (Figure 36.2), nisin exerted a similar pattern of inhibition to that observed with *E. rhusiopathiae*, except that here the complete inhibitory concentration was doubled (32 iu/ml). Simultaneously, haemolysin titres were determined and, where the survivors were able to initiate growth after initial inhibition by nisin, they were found to possess the same haemolytic power as the nisin-untreated cells.

Growth studies were then carried out to examine the effect of nisin on the growth of *L. monocytogenes* (4379 and 10357), together with the activity of nisin at different hydrogen ion concentrations and at lower temperature

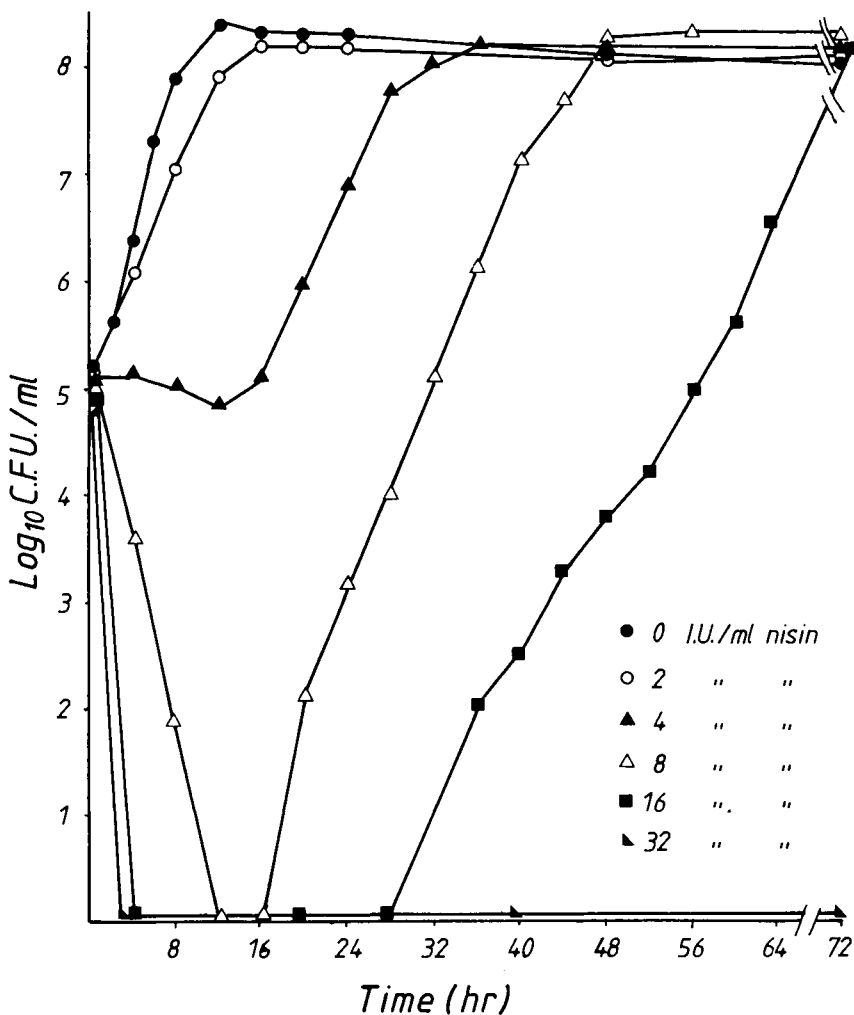


Figure 36.2 Effect of nisin on the growth of *Listeria monocytogenes* (4379) at pH 7.4 and 37°C

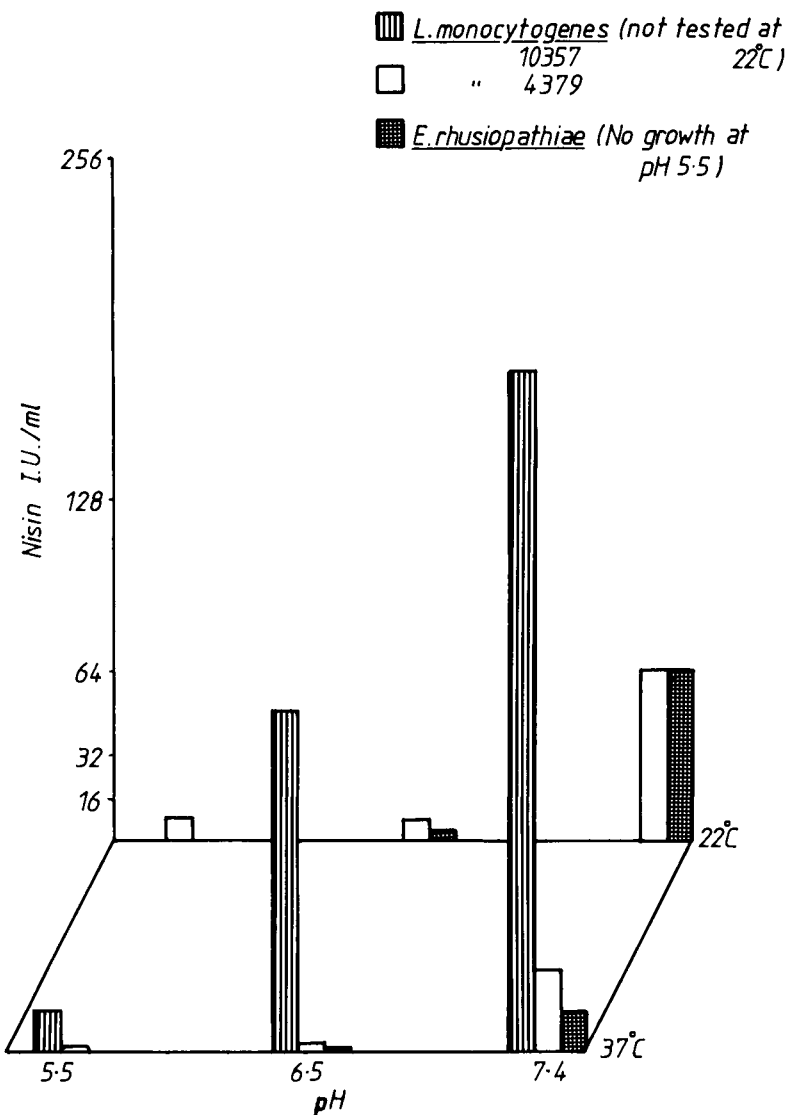


Figure 36.3 Nisin levels required for complete inhibition of *Listeria monocytogenes* (4379 and 10357) and *Erysipelothrix rhusiopathiae* (5380)

(22°C). These are summarized in *Figure 36.3*. *Listeria monocytogenes* (10357) showed higher resistance to nisin (256 iu/ml was the complete inhibitory level). At 22°C, both *E. rhusiopathiae* and *L. monocytogenes* (4379) showed a slight decrease in sensitivity to nisin, i.e. the complete inhibitory level against both organisms was 64 iu/ml. Increasing the hydrogen ion concentration in the culture medium produced remarkable enhancement in the antibiotic activity. The complete inhibitory level against both *L. monocytogenes* strains decreased by 16-fold when the initial pH of the medium was changed from

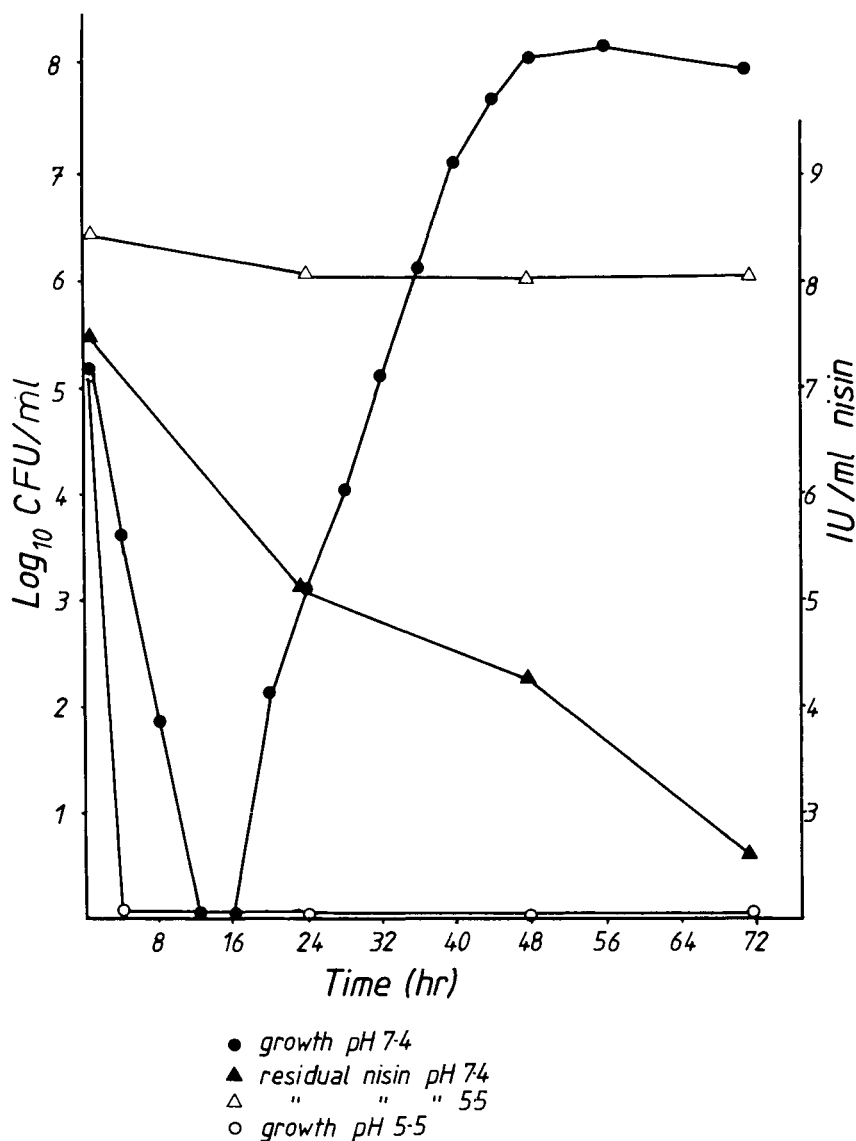


Figure 36.4 Relationship of the growth of *Listeria monocytogenes* (4379) and concentrations of nisin in the medium at 37°C

7.4 through to 5.5 and, in the case of *E. rhusiopathiae*, changing the pH from 7.4 to 6.5 decreased the complete inhibitory dose by 8-fold.

Figure 36.4 relates the growth of *L. monocytogenes* (4379) to the stability of nisin in the culture medium. Here the fact that 8 iu/ml nisin was fairly stable at pH 5.5 coincided with the complete inhibition. At pH 7.4, although growth reached a countable level when the antibiotic was reduced to 5 iu/ml, the maximum viable number was obtained, while the nisin level was still

slightly above the minimum inhibitory concentration (MIC) of 4 iu/ml (Mohamed, Seaman and Woodbine, 1981).

In other studies, the MIC and minimum bactericidal concentration (MBC) of nisin against *L. monocytogenes* was found to be stable with inoculum size ranging from 10^4 to 10^5 – 10^6 cfu/ml. Also, cells harvested from the logarithmic phase of growth (5 h old) of *L. monocytogenes* (4379) and inoculated in a medium containing different concentrations of nisin were found to be more sensitive than those harvested from the stationary phase (16 h).

Discussion

The overall sensitivity of *E. rhusiopathiae* to nisin at 37°C showed a great resemblance to that of *L. monocytogenes* (4379), but susceptibility to individual concentration reflects the slightly greater sensitivity of *E. rhusiopathiae*, e.g. 4 iu/ml of nisin produced 98.81% decline in the viable count of *E. rhusiopathiae*, while with *L. monocytogenes* there was only a possible bacteriostatic effect during the first 16 h. However, under all concentrations of nisin examined, when regrowth started *E. rhusiopathiae* grew faster than *L. monocytogenes*. This quick recovery of *E. rhusiopathiae* may be due to the difference in the rate of growth at this temperature (1.66 divisions/h is the growth rate of *E. rhusiopathiae* and that of *L. monocytogenes* is 1.5 divisions/h (Mohamed, 1982).

Changing the incubation temperature to 22°C was shown to have a slight decrease in the activity of nisin against *E. rhusiopathiae* and *L. monocytogenes*. This slight increase in the resistance of the two organisms is unlikely to be due to the lack of stability of the antibiotic, since nisin stability increases with a decrease in the temperature (Gibbs and Hurst, 1964).

The pronounced difference in susceptibility to nisin between the high and low haemolytic strains of *L. monocytogenes*, together with those reported in the previous studies (Mohamed, Seaman and Woodbine, 1981), suggest some correlation between the haemolytic power of the strain and nisin sensitivity. This difference in response to nisin is not related to any specific inactivation of the antibiotic by either the resistant or sensitive strains. However, genetic and biochemical differences between the two types of strains have been reported elsewhere (e.g. Seeliger and Schoofs, 1977; Ortel, 1977; Groves and Welshimer, 1977).

Nisin is acidic in nature and is more stable and soluble at low pH (Hall, 1966; FAO/WHO, 1969; Aplin & Barrett, 1978; Mohamed, Seaman and Woodbine, 1981). Here, nisin was also found to be more effective in inhibiting *E. rhusiopathiae* and *L. monocytogenes* at acidic pH, both at 22°C and 37°C. This increase in the antibiotic activity at low pH has been shown to occur by other authors (e.g. Campbell and Sniff, 1959; Scott and Taylor, 1981; Rayman, Aris and Hurst, 1981), and can be partially attributed to the antibiotic's stability at acidic pHs. However, Mohamed, Seaman and Woodbine (1981) reported that nisin in salt concentrations of 6%, 7% and 8%, at pH 7.4, was as stable as in acidic medium (pH 5.5), but with less activity, which indicates that the activity of nisin is distinct from its stability, confirming the explanation given by Hall (1966) where he attributed this difference in activity

between acid and alkaline condition to the polymerization or infolding of a single molecule of nisin at higher pHs.

Nisin at sub-bactericidal levels reduced the viable count to a very low level before regrowth of *E. rhusiopathiae* and *L. monocytogenes* started, reaching the same maximum viable number as the control cultures. As suggested earlier (Mohamed, Seaman and Woodbine, 1981), this could be due to the occurrence of a different sensitivity to nisin in the initial inoculum. However, it is not known whether this gradient sensitivity is an inherited property among the cells with the same ages or due to the presence of different cells of different ages, and it has been found that cells in the logarithmic phase are more sensitive than those in the stationary phase. On the other hand, the mechanism of nisin could be dependent on its concentration, i.e. small doses affect few cells and an increase in the concentration increases the number of exposed cells. However, in this respect the inoculum size was found to have a minimal effect on the MIC and MBC of nisin against *L. monocytogenes* (4379).

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ANIMAL HEALTH AND PRODUCTIVITY: DRUG DEVELOPMENT—REGULATORY INTERFACE

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New product development: the regulatory authority

The primary function of a regulatory authority is to rule upon the safety, quality and efficacy of products entering the market place. It is a primary objective of the responsible drug manufacturer to develop products which are safe, of high quality, efficacious and of commercial utility and economic viability. An essential part of this objective is the acquisition and presentation to the regulatory authority of data which demonstrates that the products are safe and efficacious. There is therefore much common ground and common purpose between the two groups; perhaps more than some recent debates may have suggested.

In the context of animal production, safety parameters encompass both the use animal and man, where man is the user of the commercial material and is also the consumer of the edible animal end-products. Safety to man similarly covers both direct and indirect contact with the product and, in the case of antibacterial agents, any consequences there may be for man as a result of the activity of the agent against, or on, environmental microorganisms transmissible to man. The safety evaluation of an antibacterial product, therefore, covers both the product *per se* and the bacteriological consequences of its use.

These interrelated biological parameters have obviously created a situation of some considerable complexity in the process leading to the demonstration of product safety; the complexity is such that it is not difficult to investigate the relationship to the point of absurdity. The interface between drug development and regulatory approval activities is therefore one which takes account of these interrelated factors and seeks to arrive at conclusions which are demonstrably correct in scientific terms and wholly justifiable in ethical terms.

Nobody would dispute the fact that regulatory authorities have a difficult task—there is seldom praise for making the right decision, but always blame for being wrong. Furthermore, there is no more complicated or contentious situation than that of the present-day use of antibacterial substances in modern animal production systems.

A wide range of antibacterial products is now used across the whole spectrum of modern animal production. The applications vary from clinical veterinary medicine on the one hand to low-level, in-feed routine supplemen-

tation for performance improvement on the other, with a correspondingly wide spectrum of routes and methods of administration to the animal.

A particular feature of the continuing developments in modern systems of intensive animal management has been the desirability and utility of herd programmes for maximizing animal health and performance. This emphasis on herd health and treatment is important; it implies herd disease prophylaxis as an integral factor of animal production; it similarly presupposes the use of in-feed additives for ensuring maximum animal performance. Such routine feed supplementation is now as essential to modern animal husbandry as are modern buildings, nutrition and management systems.

The significance of feed additives

A productive interface between the drug manufacturer and a regulatory body must, in this context, be based upon a common appreciation and understanding of the role a product is intended to serve and the environment in which it is to be used. This is obviously of fundamental importance to the whole concept of modern animal production. It has to be acknowledged, however, that there is a significant body of opinion opposed to the widespread use of antibacterial agents in agricultural practice. Thus, the strategy of use of antibacterial agents must be highly relevant to considerations of the consequences of such use.

Within the defined concept of modern animal husbandry, the 'herd treatment' situation, above all others, highlights a major difference between human and veterinary medicine. It also highlights some of the theoretical arguments which have claimed veterinary medical and nutritional usage of antibacterial agents to be a major source of the uncontrolled spread of antibiotic-resistant microorganisms in animals and man. In the broad aspects of veterinary practice and animal husbandry, there is every justification for following the principle of herd therapy or prophylaxis on both economic and humanitarian grounds; none the less, it remains a practice which is clearly distinct from conventional human therapeutic practice. Thus, there are two common systems unique to the animal production use of antibacterial agents—in-feed use of performance-enhancing agents and in-feed (or in-water) use of disease prophylactic programmes.

Antibacterial feed additives therefore have three clearly defined roles in modern intensive animal production:

- (1) Enhancing the performance of clinically 'normal' animals.
- (2) Maintaining health and performance in the face of disease and environmental stress.
- (3) Veterinary therapy of disease, particularly in the younger animal.

Such was the impact of antibiotics on animal husbandry—in the broadest sense—that there is now a wide range of antibiotics and antibacterial agents in use for disease control and the improvement of performance of all classes of farm livestock, i.e. in the three roles outlined above. It is no exaggeration to say that such agents have revolutionized modern intensive animal production systems.

The development of in-feed additives makes an interesting case history in

both practice and regulatory approval, particularly since feed is the route of administration for the great majority of non-prescription and many prescription antibacterial agents. It also highlights the chain of safety assessments now necessary to demonstrate safety to man.

A feed pre-mix, after leaving the manufacturer, can be used in three situations: in an animal feed mill for inclusion in formulated feed, or in the preparation of a vitamin/mineral/protein/supplement pre-blend for subsequent preparation of a finished feed, or directly on a 'home-mixing' farm. These are the three situations in which the highest concentration of active material (i.e. the commercial pre-mix) is handled. A typical feed additive to be used in feed at a final concentration of 50 p.p.m., with a pre-mix addition rate of 1 kg/t, will have an initial concentration of 50 000 p.p.m. of active material and will initially therefore be handled at that concentration. At the ultimate use point on the farm, feed will be handled at the intended final active concentration of 50 p.p.m. Demonstrations of safety for human operatives will therefore have to be made throughout this chain, taking into account not only the levels of active agent involved at any point, but also the sophistication and dust control properties of the equipment likely to be used. Such assessments will also have to take account of the fact that none of the equipment or operations is under the control of the product manufacturer.

One can, of course, further sub-divide the home-mixing farmer into the man with a well-engineered and efficient feed mixing system on the one hand, and the man who mixes on the floor with a shovel on the other. These *are* the end-users of the product, and safety testing and standards must acknowledge their existence. The manufacturers and the regulatory authority must therefore be able to agree a series of meaningful, use-condition experiments which will be representative of end-users as a group and will assure safety under all conditions of use.

It has been argued (Davey, 1980) that antibacterial feed additives are now essential and integral components of modern, intensive animal production. This being the case, they will obviously feature prominently in the priority lists of commercial (and academic) research and development effort. It follows, therefore, that such agents—with potential utility from growth promotant to therapeutic use—will be frequently before regulatory authorities. As they are accepted as integral components of modern systems, it follows that their usage in the field will be extensive. It is this extensive use, coupled with the unique relationship of antibacterial agents (antibiotics or not) with farm animals, the human population and the microflora and aetiology of disease of both groups, which has given rise to so much concern on the use of antibacterial agents in farm animals over the past 30 or so years. This combination of factors identifies the basic problem of development of bacterial resistance and the possible consequence for human therapeutic policy of these events in farm animals. It is this series of events which has been so significant in the development of regulatory control over the usage of antibacterial agents in farm animal production.

In the early years of feed medication, the same antibiotics were used for performance improvement, disease prophylaxis and disease therapy in farm animals. These same antibiotics were also used in human medicine, the agricultural usage being a 'spin-off' from the human field. This common usage, together with the emergence of bacterial resistance and the implications

of transferable antibiotic resistance, led to the Swann Committee, which reported in 1969, and the resulting basic changes in UK legislation. The agents first in general and continuing use were penicillin and the tetracyclines, and it was therefore inevitable that the microbiological consequences of the widespread use of these agents became the parameters of concern, judgement and ultimately legislation. From this legislation arose the current UK regulatory approval system, based upon the Medicines Act of 1968, which replaced the then existing voluntary system on 1 September 1971.

The drug development-regulatory interface under discussion is therefore that originally created by the demands of legislation and modified and developed by practical experience over the past 10-15 years.

The Swann Committee: parameters of legislation

The Swann Committee and its sequelae were, of course, the first major steps taken to give what was intended to be a constructive response to the chronic concerns which eventually crystallized over an epidemic of antibiotic-resistant *Salmonella typhimurium* in calves. The intent was that elimination of uncontrolled antibiotic usage and the categorization and differentiation of 'feed' antibiotics for routine non-prescription use in farm animals would reduce the incidence of antibiotic-resistant R-factor-carrying strains of organisms such as *S. typhimurium*, which are potentially pathogenic to man and which under conditions of multiple antibiotic resistance could present severe theoretical therapeutic problems.

This is the situation in which today's regulatory authority and commercial research and development operations find themselves, and it is against this background that new strategies have developed. It should be noted, however, that the first 10 years of 'Swann-type' control and categorization of antibiotics for agricultural use in the UK has not eliminated concerns at either the wholly scientific or consumer levels. Hence the continuing demands for more research and tighter control of this area of antibiotic usage.

The end objective of the specific legislation in this context is, as it always has been, to protect the efficacy and integrity of antibacterial agents used in human medicine. This is an objective as important to the product manufacturer and the product user as it is to the regulatory authority or the consumer. In some instances, specific products or active agents may be reserved strictly for human use, so the question of agricultural utility does not arise. In other instances of common usage, which would be almost inevitably in veterinary ethical circumstances, the case for such use will be judged on its merits.

Although many of the new agents in current animal production use are not used in human medicine, and are not chemically or biologically related to agents which are, concern still exists that wide usage of agricultural antibiotics in general terms could prejudice human therapy either directly or indirectly. In current terms, therefore, the producer of a new antibiotic agent must take account of these views in his active compound selection and testing programmes, while the same terms of reference will be prominent in the review procedure carried out by the regulatory authorities, and in the discussions which will have preceded formal submissions by the manufacturer to the regulatory authority.

Reviewing the situation as it now is, one point is apparent at the outset: there is a wide spectrum of essential antibiotic and antibacterial product usage in animal husbandry. To meet the demands of the industry, there are many new and biologically potent agents in agricultural use and no doubt in development. The majority of them will see no use in human medicine, but they are none the less potent, effective agents. They present both drug manufacturers and the regulatory authority with challenging decisions. It is difficult to see how these challenges can be successfully met if a divisive approach is made towards an evaluation of the rationale, safety and efficacy of their use.

Determination of the efficacy of antibacterial products is generally a matter of objective judgement and statistical appraisal of data against pre-set criteria, and as such does not normally raise any unusual problems of principle or ethical concern. Such demonstrations of efficacy in the context of animal health use of antibacterial agents emphasizes the diversity of the applied usage of such products.

Disease therapy is conventional by medical standards (and it is here that the common use of agents also employed in human medicine is most likely to be found), and judgement of therapeutic success is based on conventional methods of data analysis. Prevention of disease, particularly in the context of intensive animal production, is a procedure developed within the overall management system being employed at any particular unit; prophylactic systems can therefore be tailored, if necessary, to meet individual situations and circumstances. This is not a common procedure in human medicine. Judgement of efficacy here, of course, is a combination of maintained health and performance, the latter being in some measure dependent upon the former. It is, of course, important to remember in all these analyses that performance is, at the end of the calculation, the parameter of primary concern, whether it be measured in terms of meat, eggs or milk production. A healthy animal is a potentially productive animal; a sick animal is not productive and, moreover, is very often an epidemiological menace to other animals. This is the basic rationale for the use of animal health programmes. Finally, the use of antibacterial agents to improve performance has now developed to the situation we see today of a range of 'speciality' antibiotics solely for improvements in performance—notably, in more recent developments, improvements in feed conversion.

All these improvements in animal health and productivity can be quantified and their economic value determined. Efficacy can therefore be determined in health and production terms and in cost-benefit values. It is these parameters which can then be assessed in the light of broad safety evaluation.

Many aspects of the safety evaluation of antibiotics are readily dealt with by conventional and well-established procedures and parameters, leaving for special consideration those issues of safety peculiar to the in-use properties of antibiotics and antibacterial agents in general. These can be summarized as:

- (1) Effect on the microflora of the host animal (and the significance of any such effects).
- (2) Interactions (direct or indirect) with human therapeutic antibiotics.
- (3) The significance of the antibacterial activity of (a) tissue residues and (b) the product or its metabolites excreted into the environment.

Effects on the antibiotic resistance of the host microflora, together with interactions with human therapeutic antibiotics, represent the key issues in such a review. It is of course these very points which are the most difficult to define and identify in specific terms. They will almost inevitably require extensive field studies, very much orientated to the ecology of the gut flora as it is influenced by the projected commercial usage of the antibacterial agent.

It has often proved difficult to lay down other than very general guidelines towards the evaluation of the possible resistance problems following the use of antibacterial agents, such that it is almost inevitable that each particular situation will have to be judged on its own merits. This may well mean that a set of criteria are developed for an individual agent. If so, then this is arguably not a bad thing—for that product. Review under such terms would enable a potential product to be assessed in its own environment and against its own pattern of usage, enabling environmentally relevant guidelines to be set and meaningful parameters of judgement established. However, in spite of the advantages for the product of being judged against its own definitive criteria, this judgement as a whole must still take place within consistent overall guidelines, which must none the less be sufficiently flexible to allow the place of a new product and parameters for its assessment to be definitively established. It must also be acknowledged that the difference of opinions over the philosophy of usage of antibacterial agents often makes the establishment of definitive criteria exceedingly difficult. Hence, the interaction between the manufacturer and the regulatory authority must be particularly close and sympathetic, both in terms of overall general guidelines and also specific parameters for individual products.

In the general context of potential interaction with 'human' antibiotics, the interactive points for definitive evaluation need to be clearly established. Is the drug likely to reach the human population—via, say, milk or meat? (If so, what are the likely consequences, and how can these be determined?) Is there a hypothetical—or real—possibility of cross-resistance with human therapeutic antibiotics? Does this cross-resistance (if it occurs) contain transferable resistance factors? (How can these factors be measured, and the risks evaluated?) What components of the animal microflora are affected? Are they potentially transmissible to man? And so on. The factors to note here are, again, the speculative nature of these interactive points. In very few cases is it possible to say, 'If so-and-so happens, it is *known that* . . . '.

The majority of investigations directed towards these points can, of course, only be carried out in depth in the field situation. This therefore implies that initial regulatory approval, where appropriate, has been given for such field work to be carried out. This, in itself, implies a degree of act of faith by both the drug producer and the regulatory authority. It is possible to go so far by *in vitro* and small-scale 'in-house' studies, but the product must, in the final analysis, be evaluated in the field under conditions of actual use based on laboratory and limited *in vivo* data. It may well be that the only ultimately satisfactory scale of evaluation in terms of time and numbers will come from commercial use, in which case some form of post-marketing approval, bacteriological monitoring programme would have been determined to be appropriate. Again, such a field programme must be the result of close interactions between the manufacturer and the regulatory authority.

Environmental data and the regulatory authority

There has, over the past few years, been considerable talk of the desirability of field monitoring programmes to provide background information on bacterial antibiotic resistance in the farm animal—and human—environment. Some such information is available, but what it tends to lack are the elements of cause and effect. Resistance to, for example, tetracycline in the gut flora of animals and humans is acknowledged to be widespread. This phenomenon has been ascribed to the widespread and allegedly indiscriminate use of tetracyclines (including low-level in-feed use) in farm animals but, as Richmond (1981) has calculated, something like one in every 150 people in the UK has a tetracycline-resistant gut flora solely due to the medical therapeutic use of that class of antibiotic. Similarly, Gedek (1979a) has shown that the *therapeutic* use of antibiotics in veterinary medicine can produce a level of environmental resistance at least equal to that produced by low-level in-feed use. Categorization of antibiotics such as the tetracyclines as therapeutic antibiotics for use under veterinary control only has therefore not resolved this particular issue; it remains the backdrop to current deliberations and to be interpreted, in the context of current antibiotic usage, in somewhat speculative terms. The fact that there has not been a significant reduction in tetracycline resistance since these antibiotics were transferred to veterinary control may be due to continual selection pressure from the ethical (human and veterinary) use of tetracyclines, or the fact that the resistant strains are now able to co-exist with sensitive strains in their environment with no ecological disadvantages. There is, however, an acknowledged lack of relevant data which properly relates contemporary animal health usage of antibiotics with the patterns and real significance of antibiotic resistance in the enteric flora of farm animals and man.

It has been pointed out many times that regulatory authorities (the Veterinary Products Committee in the case of the UK), do not have the money or resources, or indeed the remit, to undertake antibiotic resistance monitoring. This is true, but the Veterinary Products Committee, together with its Secretariat and associated committees, does, like other regulatory bodies, have considerable experience in the field and must be very well situated to see the way ahead in its own interpretation of scientific advance, commercial need and public protection. Such a task could perhaps be undertaken with other interested sections of the agricultural industry—drug manufacturers, veterinary surgeons, nutritionists, etc. This implies a rather wider role for a regulatory authority than exists at present, but if one reflects on the number of recent papers, symposia, etc., which have discussed the problem of antibiotic resistance in the agricultural environment and still concluded with the question 'Where do we go from here?', then such an initiative is clearly overdue. Apart from the desirability of such data being generally available, they would be of unique value to regulatory bodies as the base for developing strategic guidelines towards a general policy for antibiotic usage. The Food and Drug Administration in the USA, as an example, now has a remit to develop such data.

Contemporary legislation

This then is the background against which the manufacturer develops his new products and the regulatory authority seeks to satisfy itself as to product safety, quality and efficacy. It is not difficult to see the inherent problems faced by both groups, particularly if the data base used for comparative judgement is heavily biased by the older, broad spectrum antibiotics, where virtually any dose level given by any route for any purpose has been shown to produce long-term resistance in the enteric microflora.

Having made the case for animal health antibiotic products to be judged in their own environment, there is a particular facet of product approval which deserves further comment, i.e. the question of exactly what is approved: formulated product or active drug principle only. As the situation stands at present, clearance for feed additives within the European Economic Community (Annex I) under Directive 70/524 is granted on the basis of the submission and approval of a full regulatory dossier describing a formulated product which has been extensively evaluated in the laboratory and in the field. The formulation of the product may be relatively simple, or it may be a complex system with crucial relevance to both drug safety and efficacy. However, approval for use appears in the Annexes I/II lists as the active principle only, with no reference to the formulation of the product or any essential product properties related to formulation. This is in clear distinction to the procedures in most individual countries and can only be seen as unscientific and unreasonable, if not irresponsible. It is an open door to generic products which can, at the moment, at least from the regulatory point of view, contain the same (claimed) active principle, but which, by virtue of inadequate or deficient formulation, may be quite different in practical use from the original 'parent' product which received regulatory approval. This surely cannot be the conscious intent of any responsible regulatory authority.

If specific formulation is essential to safety, efficacy and quality (e.g. stability), and the product dossier will certainly highlight this, then approval should acknowledge the fact. This should be as fundamental an axiom of all approval philosophy as it necessarily is of the original drug development and evaluation process. This factor will certainly assume greater significance, since many products are now dependent upon formulation for their optimal in-use activity; modern formulation technology is becoming a very significant, sometimes determinate, tool in new product development. This new technology is being actively applied to safety and efficacy; safety and efficacy judgements and consequent regulatory approval must therefore take account of this.

It is therefore essential that legislation controlling the introduction and use of new products—be they antibiotic or not—should be kept under regular review and, within the limits of accepted safety factors, should be alive and responsive to advances in technology. Modern animal production is a vital and dynamic industry, likewise the related industries that serve it; they deserve to be recognized as such.

In this context, it is arguable that Article 6 (2 A.d.) of EEC Directive 70/524 (the basis of Annex I and Annex II clearance in Europe), as presently worded and interpreted (... products with clinically useful antibacterial activity may not be approved for free sale Annex I use ...), is somewhat

irrelevant and indeed restrictive to modern animal management systems. It is argued—with some validity—that the directive, which came into force 13 years ago, is outdated and has for some time required revision to reflect the growth in modern animal production systems. There are now, for example, a number of antimicrobial products with properties eminently suitable for disease prophylaxis, but which cannot be used as such on a routine basis because of the restrictive nature of Directive 70/524. A case can certainly be made for amendments in legislation to permit the routine use of products such as these *which were produced for just this purpose*. The Regulatory Authorities *per se* may not have the power to amend such legislation, but they are in at least as good a position as anybody to identify unnecessarily restrictive and anachronistic provisions and to propose more relevant and acceptable guidelines to the appropriate legislation. By this means, they could guide and influence the development and strategy of the use of antibiotics in the agricultural environment in both general and specific terms. Such recommendations from bodies with unrivalled knowledge (of both approved and rejected products) and complete impartiality, would be of obvious value to those who frame the regulations under which product approvals are given. It should also be possible for this to be done without any breach of confidence of the technical data contained in product dossiers which would, of necessity, form the basis of the case for legislative updating.

Recently, an interesting new facet has emerged in the antibiotic resistance story. This is the demonstration that some feed additives will 'cure' enteric microorganisms of antibiotic resistance (particularly multiple resistance) and possession of R-factors. Several examples of this have been demonstrated. Flavomycin (Pohl, Laub-Kupersztejn and Thomas, 1975), zinc bacitracin (Walton and Laerdal, 1980), anthelmintic agents (Walton, 1972) and carbadox (Gedek, 1979b; Davey, 1980). These observations represent a possible breakthrough in the circle of conventional antibiotic usage and resistance. If these agents will, under practical conditions, reduce a pre-existing level of multiple resistance and R-factor transfer, or prevent their development, then this is surely a principle which should be exploited in practice and which should find favour with regulatory authorities. A broadly based scientific initiative here would certainly acknowledge the significance which is attached to all aspects of the control of multiple transferable antibiotic resistance in farm animals.

The wider regulatory interface

The interface discussed so far concerns the direct manufacturer: regulatory authority contact in the context of the approval and introduction of a product to practical use in the field. However, the outcome of deliberations on safety and efficacy must obviously be reflected in field usage of the product. So far as the consumer is concerned, the question of tissue residues and withdrawal periods are particularly significant in this respect. Product clearance is based, where appropriate, on withdrawal periods and, while safety margins are built into the appropriate calculations upon which withdrawal periods are calculated, it is obviously incumbent upon the user to follow instructions for use precisely. This is another area where common ground between the regulatory

authority and the product manufacturer is important, and the 'total concept' review of a product essential, to ensure that recommendations for use are compatible with the activity of the product and are such that they can, and will, be followed in on-farm use. While this appears obvious, it is also the key to the successful and safe use of any product, and it is essential that both product manufacturer and regulatory authority are completely satisfied that proper usage can be assured.

At the other extreme, it is equally important to recognize and acknowledge the influence of regulatory authorities upon the strategy of industrial research and development. It would obviously be unwise in the extreme to take a potential product to the point of field trial without careful assessment of its properties in the regulatory context. It is obviously prudent to bring such considerations to bear at as early a stage of potential product selection as possible. A reputable manufacturer will first satisfy himself, rather than a regulatory authority, that his products are safe and effective. The thesis of a common interest in demonstrating safety and efficacy would not be advanced by a manufacturer seeking to use the regulatory authority as his own conscience. It is equally prudent to continue to assess the properties of the agent throughout the research and development programme in the light of regulatory circumstances, since these could well change during the life of such a programme. An interacting relationship with the regulatory authority will enable key points to be established where decisions based upon regulatory considerations are to be made. This is particularly the case when new biological entities with novel biological profiles are isolated and identified as early product candidates. It may well be that existing legislation—being established on older criteria—might not permit the use of such an agent or might not permit the full expression of its properties. In such a situation the regulatory authority must have the freedom from restrictive legislation to be able to review and assess the product in its own right and in the light of modern scientific knowledge and advances. The initiative will come from the industrial research effort, but it can properly expect to find a review body alive and responsive to the significance of advances in new technology and applications.

It is alleged from time to time that regulatory authorities are restrictive to new product technology. In overall terms, this should not be true. Their brief is to establish safety, quality and efficacy and to be satisfied with nothing less or to demand anything more without very good reason. To do this, they rely almost exclusively on data provided by the product manufacturer or sponsor, together with their own experience and judgement. It is certain that over the years they have had a very positive effect upon the direction, rationale and comprehensiveness of industrial research and development. At their requirement, new techniques over a range of disciplines have been developed, from advanced assay methods to new principles of environmental safety testing. Without doubt, many new comprehensive and objective testing systems have been introduced both for safety and efficacy evaluation purposes. Obviously, many of these new systems would have been developed by industry, whether or not the current statutory regulatory controls were in existence but, none the less, there is now a more co-ordinated and systematic approach

to objective new product evaluation than would be the case in a commercial free-for-all, of which the less responsible elements would no doubt have taken advantage. However, there are a number of instances where, on a worldwide basis, regulatory authorities have taken different positions in relation to the approval of the same product, or type of product. This therefore gives rise to situations where a product, available in some countries, remains unapproved in others. Potential product users in the latter countries will obviously feel that their regulatory authority is being restrictive and depriving them of products which are in common use elsewhere. Antibacterial agents, for both general performance improvements and clinical disease control, feature in such a worldwide comparison of product approval. In some instances, such situations have been of relatively short duration pending the provision of additional data, but there are contemporary examples where it is obvious that fundamental differences in approval philosophy exist between some regulatory authorities, which are reflected in international product availability or in approved indications and methods of use.

Regulatory authorities, however, have been properly restrictive in respect of inadequately tested or poorly manufactured products. They have also been restrictive in terms of products whose efficacy is non-existent or whose safety is suspect. Identification and control of such products is one of the more important tasks for which regulatory authorities were created.

The question of approval of a new product in a number of countries is an issue highlighted by, but not exclusive to, Europe. Within the European Economic Community, the various EEC directives, in particular, the Veterinary Medicinal Directives 81/851 and 81/852 which were planned to take effect by September 1983, are beginning to draw regulatory requirements into a common system, but for a number of years submissions throughout Europe have been exceedingly complicated. Holland, for example, did not require approval of veterinary ethical products, although that position will change this year. Eire had a voluntary scheme, but will introduce a mandatory scheme this year. Approval of submissions in France and Germany are very dependent upon a number of 'expertises' prepared by independent experts in, for example, safety, efficacy or chemistry and pharmacy. The Scandinavian countries tend to have more exacting requirements for acceptable tissue residue levels than do other countries. Recent developments, however, strongly suggest that there is positive and very desirable movement towards a common European position. In the context of antibacterial agents, the Food and Drug Administration in the USA is one of the few regulatory bodies which has not moved to a 'Swann-like' position. The FDA current stance is that more data is required to determine the existing levels of antibiotic resistance and to review the practical significance of the findings, before considering a new regulatory position in relation to antibacterial agents.

Registration and approval of products in countries where these differences in regulatory philosophy exist has, of course, been a testing exercise and will evidently continue to be so for some time to come. This clearly highlights the significance of the drug development-regulatory interface and the determinative role that this interaction plays not only in the development of individual products, but in the development of the animal health industry as a whole.

Summary

To produce a brief summary of a subject such as this is, of course, difficult and certain to do some aspects the injustice of omission. However, there are a number of key issues which form the skeleton of the regulatory system. In the majority of countries, data covering at least safety, quality and efficacy of new products must be submitted to some form of technical review body for approval prior to marketing. That body must review the data and report as to its adequacy to satisfy these key issues. That is the system prescribed by legislation. How the system operates in detail obviously varies, both within differing legislative frameworks and also with the philosophy of those who direct the regulatory activities. What is looked for, of course, is a dynamic and responsive system, alive to the needs of agriculture and to the scientific purposes of modern animal management systems, balancing these with the responsibility of user and consumer protection.

Equally, new products submitted for approval should be supported by adequate, well-constructed and well-presented data, based on experimentation of the highest standard. An industry which seeks high standards and technologically responsive thinking within its regulatory activities, should be the first to set those standards and let it be seen that they are the standards it expects and can maintain itself.

These are indeed high standards, but nothing less should be expected of a multidisciplinary and complex industry which, in its broadest terms, is responsible for both the health and welfare of man and animals.

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PENICILLIN HYPERSENSITIVITY—IS MILK A SIGNIFICANT HAZARD?

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Introduction

It has been recognized for many years that hidden sources of penicillin might represent a hazard to penicillin-allergic patients. Coleman and Siegel (1955) were the first to draw attention to this possibility by reporting a case in which penicillin contaminating a sterilized syringe was implicated in the development of anaphylactic symptoms in a patient receiving a testosterone injection. The patient had previously developed generalized allergic reactions after oral therapy with penicillin and also following the use of penicillin skin tests. Bierlein (1956) and Siegel (1959) drew attention to other hidden sources of penicillin, including ingestion of penicillin in vaccines and in contaminated milk.

The purpose of this chapter is to assess the significance of low levels of penicillins in milk in terms of risk to penicillin-allergic patients and of sensitization *de novo*. Three basic questions are addressed.

- (1) Does what is known of the fundamental processes of penicillin allergy, in terms of immunochemical pathways and penicillin-derived allergens, provide a mechanistic framework within which a role can be established for penicillin in milk?
- (2) Is there evidence that low levels of penicillin or penicillin-related substances can act as allergens by the oral route?
- (3) What is the clinical evidence of risk to individuals or of public health hazard?

Basic mechanisms of penicillin allergy

Penicillin allergy resulting from the therapeutic administration of penicillins is well documented. *Table 38.1* summarizes the main characteristics of penicillin-induced allergic reactions on the basis of underlying mechanism. IgE antibody-mediated reactions are the most significant. The mechanism by which these reactions can lead to acute clinical episodes, most commonly urticarial lesions but, in rare instances, life-threatening anaphylactic re-

Table 38.1 IMMUNOLOGICAL MECHANISMS INVOLVED IN PENICILLIN ALLERGY

Type	Immunological mechanism	Manifestation induced by penicillin
I	Release of vasoactive amines from mast cells following interaction between IgE antibody and allergen on surface	Urticaria, angio-oedema, anaphylactic reactions, rapid in onset
II	Interaction of IgM or IgG antibody with penicilloylated erythrocytes with uptake of complement	Haemolytic anaemia
III	Inflammatory reactions due to deposition of antigen-antibody complexes in vascular system and skin	Serum sickness syndrome. Drug induced fever. Possibly erythematous rashes
IV	Generation of lymphokines or cytotoxicity by T lymphocytes in absence of free antibody	Contact dermatitis

Source: after Coombs and Gell (1975).

actions, is established. It involves the release of chemical mediators of inflammation from the mast cell, a cell of the granulocyte series, which occupies a pivotal role in these acute, immediate hypersensitivity reactions. Other clinical syndromes may also be relevant to the issue of penicillin allergy resulting from ingestion of milk containing penicillin residues. In particular, it is important to consider the possibility that immune complexes could be formed and initiate both cutaneous reactions and other symptoms reminiscent of serum sickness. Contact allergic reactions are unlikely to be of significance in this context, but may arise as a consequence of handling penicillin preparations for intramammary use.

It is clear that not only are the penicillins capable of initiating immunological reactions, but that there is marked heterogeneity in the immune response to penicillins and consequently in clinical responses also (Levine, 1966). The immunochemical pathways involved in the formation of penicillin-derived antigens are well documented and they focus essentially on the concept that the ability of low molecular weight chemical compounds to stimulate a specific immune response is a direct function of reactivity with protein amino groups or other nucleophiles. Thus, the hapten theory of drug immunogenicity states that covalent reaction between the drug or derivative of it and the macromolecule is required before an immunological response can be generated (reviewed in Dewdney, 1979). The penicillins are capable of this protein reactivity.

Figure 38.1 shows that reaction can occur at physiological pH between the β -lactam carbonyl group of the penicillin and protein amino groups to form the penicilloyl determinant. The major population of antibodies generated as a result of penicillin administration in man and animals is directed against this penicilloyl determinant underlining its importance as a potential allergen. The same determinant can also be formed by reaction of penicillic acid, a breakdown product of penicillin, with protein amino groups, as shown in *Figure 38.1*. It is of interest that penicilloic acid, the major degradation product of penicillin, does not have the ability to react covalently with protein amino groups, although there is some evidence of reaction with thiol groups of proteins (*Figure 38.2*). It has been claimed that penicilloic acid can act as a minor determinant in penicillin allergy, but the precise mechanism has not

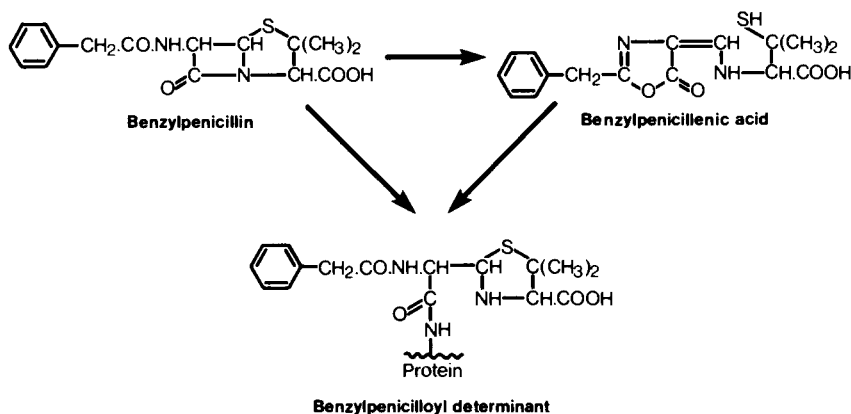


Figure 38.1

been clarified. This point will be referred to later, in the discussion of the use of penicillinase in the prophylaxis and treatment of penicillin-allergic patients.

Thus, mechanisms can be put forward to explain the ability of penicillins to stimulate immune responses. It is worth noting that these immunochemical pathways have been established primarily using benzylpenicillin as a model. It may not be appropriate to extrapolate either qualitatively or quantitatively to the range of semisynthetic penicillins now available. This is also true of the cephalosporins. These too are β -lactam-containing antibiotics and share with the penicillins protein reactivity through the β -lactam carbonyl group. However, there are significant differences and extrapolation is not always possible. An earlier review addresses a number of these immunochemical points (Dewdney, 1977).

Within this general framework, routes to the formation of penicillin-related allergens, consequent upon the use of intramammary penicillin preparations to control bovine mastitis, can be defined. Table 38.2 lists these putative

Table 38.2 PUTATIVE PENICILLIN-DERIVED ALLERGENS IN MILK

<i>Preformed or complete allergens</i>	<i>Pro-allergens or haptens</i>
Penicilloylated bovine proteins	Penicillin
Penicillin-derived polymers	Penicillenic acids
	Penicilloic acid

allergens, and draws attention to the fact that they may form *in vivo* following absorption of penicillin or its degradation products from the gastrointestinal tract, or they may be preformed and present in ingested milk.

There is no doubt that penicillin and its degradation products can be found in milk following therapy for mastitis. There is, therefore, the theoretical risk that sensitization *de novo* or reactions in the already sensitized patient could occur. Quantitative arguments which assess the probability of such events taking place are presented in the next section. Such arguments are based on the related factors of the amount of penicillin likely to be ingested in this form and on the concentration of penicilloylated autologous proteins so formed. As will be discussed, such substituted proteins have been detected in

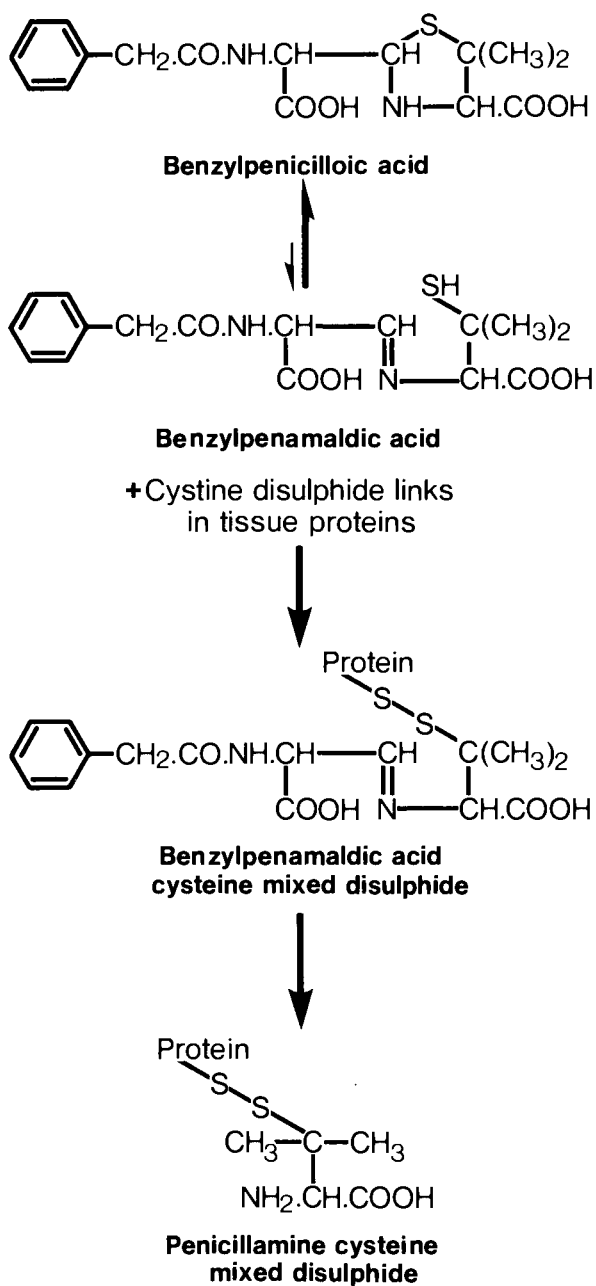


Figure 38.2

experimental studies (Wal, 1980), but only following extremely high intramuscular doses of penicillin, not following ingestion of penicillin in milk.

It is worth drawing attention to further studies by Wal (1980), although they are unlikely to alter the overall quantitative assessment of risk. In these studies it was shown that there may be underestimation of the degree of penicilloylation of autologous protein by radioimmunoassays because some penicilloyl groups may be masked and only exposed on enzymic degradation of the protein. It is conceivable that this could lead to an individual being exposed to penicilloylated proteins over the period of normal protein catabolism *in vivo*, but it remains to be established that protracted penicillin allergic reactions are mediated in this way.

Preformed allergens, in the form of penicilloylated bovine proteins, have been detected in milk obtained shortly after the infusion of high levels of penicillin into the teat canals of the bovine mammary gland. Quantitative aspects of this study (Wal and Bories, 1975) are discussed later, but it is important to understand that for these penicilloylated bovine proteins to be allergens, they must first be absorbed from the gastrointestinal tract following milk ingestion. No data are available on the absorption of penicilloylated proteins, but studies on food allergy show quite clearly that intact protein can be absorbed through even the healthy gut mucosa. This may be exaggerated in the infant, perhaps due to deficiency of secretory IgA antibody, and in adults consequent upon some inflammatory gut disorder. The major milk protein allergens, α -lactalbumin, β -lactoglobulin and bovine serum albumin, can be absorbed by the human gut under these circumstances and may give rise to cows' milk allergy (Lessof and Buisseret, 1981). It can be assumed, therefore, that penicilloylated proteins could be similarly absorbed. Whether low levels of such penicilloylated proteins could be responsible for penicillin allergy is quite unknown, but quantitative arguments will be presented shortly.

A further source of preformed allergens to be considered are the dimers, oligomers and polymers (for review see Dewdney, 1977). It has been shown that penicillins can polymerize in solution and routes to the formation of polymers, and their structures, have been described. In animals, it can be shown that these substances are antigenic, but their role in penicillin allergy has not been established. In general, it would seem that at least the larger polymeric forms could be expected to give allergic reactions in penicillin-allergic patients without the necessary requirement of prior protein reactivity. No information is available on the presence of polymeric forms in bovine milk and it is difficult to speculate on this question in the absence of knowledge of penicillin concentrations and the pH of the environment in the udder, both of which would have a significant influence on rates of polymer formation.

Quantitative aspects of primary sensitization and challenge

It will be apparent that it is possible to define, and in some circumstances to measure, potential allergens derived from penicillin in milk supplies. The critical factor to be established now is the possible importance of these allergens from a quantitative aspect. There is no doubt that total penicillin exposure of any individual through milk ingestion is low. Market research

figures can be used to attempt to quantify this exposure. For example, in 1980, the average medical use of the penicillin group of antibiotics represented approximately 2.4 g per individual. This contrasts with the figure of 1.3 mg that theoretically could be ingested in milk by an individual in a year, assuming an average yearly consumption of 133 l milk and a maximum penicillin level of 0.01 µg/ml. A person drinking 250 ml milk per day could receive a maximum of 2.5 µg penicillin; a patient receiving oral therapy would receive, at minimum, 1–2 g.

The comparative risk from these two sources of penicillin exposure seems obvious, but it is important to assess that risk both from the point of view of primary sensitization and from risk of eliciting reactions in sensitized patients.

PRIMARY SENSITIZATION

Preformed allergens

Analysis of the experimental data of Wal and Bories (1975) provides a basis for the quantification of penicilloylated proteins in cows' milk. In their study, 600 mg of benzylpenicillin* were infused into each mammary gland quarter and penicilloyl residues were measured by radioimmunoassay in milk obtained twice daily for 4 days. The maximum level of penicilloyl residues recorded was in the first milking after treatment and was 1.5 µg/g milk. Assuming that the penicilloyl residues were covalently bound to bovine albumin, the degree of penicilloylation would thus be very low, at 0.009 groups per protein molecule. This figure is derived by the following calculation. If the assumption is made that milk contains approximately 3.3 g% protein, then 1 g milk contains 33 mg protein. If the assumption is then made that the protein is albumin of mol. wt 68 000 (the calculation would not be significantly different for the other major milk proteins), then in 1 g milk there are 490 nmol protein (0.033/68 000). The 1 g milk contained 1.5 µg penicilloyl residues (Wal and Bories, 1975) of mol. wt 335 as free acid, or 4.5 nmol (0.000 001 5/335). Since the penicilloyl residues must be bound to protein amino groups, the 490 nmol protein contain 4.5 nmol penicilloyl and 1 mol protein would contain 0.009 mol penicilloyl. A substitution rate of this order means that 1 molecule protein in 111 would contain 1 penicilloyl group and the remaining molecules would be non-penicilloylated. The number containing more than 1 penicilloyl group would be exceedingly low and cannot be calculated.

Again, on the assumption that milk contains approximately 3.3 g% protein, the 1.5 µg penicilloyl residue assayed would be contained in 33 mg protein or in 1 ml milk. Thus, overall, 1 ml milk could contain 300 $[33 \times 1000/111]$ µg penicilloylated protein and a person drinking 250 ml milk could receive, on this calculation, 75 mg penicilloylated protein.

It might be assumed that this level of protein could represent an immunogenic dose for man were it not for three factors militating against such a role.

The first of these factors is the extremely low level of substitution of the

* Dosages and concentrations of penicillin quoted in this chapter have been derived by the authors from figures in the original literature in units on the basis of 1.67 units of benzylpenicillin = 1 µg, or 1.0 unit = 1 µg for the procaine salt.

protein carrier by penicilloyl groups. Numerous studies have shown that lightly substituted conjugates are very poorly, if at all, immunogenic with respect to responses specific for the hapten group. Kristofferson, Ahlstedt and Svård (1977), for example, found that the epitope density of a variety of penicilloyl protein conjugates had a very significant influence on immunogenicity. Conjugates carrying an average 0.6 penicilloyl residues per molecule protein failed to induce penicilloyl specific antibody, whereas those of higher levels of substitution did so readily.

At the calculated substitution level of 0.009 groups per protein molecule arising from penicillin in milk, it seems therefore unlikely that hapten-specific antibody would be generated.

It should be noted that, in the experimental studies from which these calculations are derived, an intramammary dose of 600 mg infused into each teat canal was used. This is in excess of normal therapeutic doses of 100–300 mg. It seems probable, therefore, that in clinical practice even these substitution levels would not be achieved.

The second factor which must be taken into account, in assessing the immunological hazard of these preformed penicilloyl conjugates in milk, is that they are ingested by man. There is very little quantitative data in the literature on the immunogenic dose of a protein or a substituted protein by the oral route. Reference to the literature on food allergy, and in particular to cows' milk allergy, is helpful in that the syndrome clearly demonstrates the possibility of absorption of proteins through the gut mucosa and the development of an antibody response to them, but quantitative considerations are, of course, totally different.

Some perspective might be gained by consideration of other situations involving low-dose immunization. In the inhalant allergic conditions, for example, pollen hayfever, it is likely that the total inhaled allergen dose in a pollen season is of the order of 100 µg and this is clearly sufficient when repeated seasonally to sensitize patients (Platts-Mills, 1981). Moreover, studies in animals have consistently shown that repeated low-dose immunization schedules are the most effective way of producing IgE antibody responses (Levine and Vaz, 1970) and it may be that this is true also of man, but in these studies parenteral routes were used.

The third factor relates to the kinetics of the appearance of penicilloylated proteins in milk after mastitis therapy. Using a radioimmunoassay which detects penicilloyl groups, it has been shown that penicilloylated proteins in milk follow a similar elimination curve to that of penicillin itself (Wal and Bories, 1975). In this experimental study, when peak concentrations of penicilloyl residues were 1.5 µg/ml, penicillin titres were 22.5 µg/ml. By the eighth, 12-hourly milking, the penicilloyl residue was almost undetectable at 0.002 µg/ml, but the penicillin level remained above the acceptable level of 0.01 µg/ml. Thus, a milking-out time appropriate for the penicillin residues would be more than adequate to eliminate the possibility of penicilloyl residues at significant concentration being present.

It is difficult, therefore, against this background, to sustain the view that the low levels of preformed allergens present in bovine milk after very high-dose treatment represent a significant hazard for man with respect to sensitization capability.

Pro-allergens or haptens

It is even less likely that penicillin *per se*, acting as a pro-allergen or hapten, could be a significant immunogenic stimulus. It is known that the degree of penicilloylation achieved *in vivo* by penicillin therapy is low. In an experimental study by Wal (1980), for example, in which a very high intramuscular dose of benzylpenicillin, 6 g, was administered to a pig, covalently bound penicilloyl residues could be detected at a peak level of little more than 10 ng/mg albumin, representing 0.002 penicilloyl groups per protein molecule.

The lack of immunogenicity of such lightly substituted proteins has already been discussed and in this situation there is a further factor which would minimize the immunogenic challenge of such conjugates. It is known that substituted autologous proteins are significantly less immunogenic than are equally substituted foreign proteins. For example, it has been shown recently that penicilloylated autologous proteins are very poor immunogens, even using immunizing schedules designed for maximum response (Ahlstedt, Kristofferson and Pettersson, 1979). Bearing in mind the very low oral dose that could arise from penicillin at the accepted level in milk, that is some 2.5 µg in a 250 ml drink and the consequently minimal levels of penicilloylated autologous proteins that could be generated, the risk of sensitization can essentially be ruled out.

REACTIONS IN SENSITIZED INDIVIDUALS

Preformed allergens

Much the same considerations apply to the role of penicilloylated proteins in eliciting reactions in sensitized individuals. The extremely low level of hapten substitution likely to be generated means that the number of even monosubstituted proteins is low and of multisubstituted, orders of magnitude lower. A minimum of disubstitution is a general requirement for a challenging antigen (Levine, 1965; Kristofferson, Ahlstedt and Svård, 1977) and, in these circumstances, is not achieved.

Pro-allergens or haptens

Regarding penicillin itself, it is known that oral therapy can result in generalized allergic and anaphylactic reactions in penicillin-allergic patients, but the incidence of such reactions is relatively low. Idsøe *et al.* (1968), in an analysis of fatal anaphylactic reactions, found that only 2% were elicited by oral therapy. A more recent review (Becker, 1976) of world literature records only seven cases of serious reactions after oral penicillins. It is impossible to judge from these cases the minimum dose of penicillin which would have elicited reactions in these patients, as all had received full therapeutic doses, within the range of 0.25–1 g.

There are several clinical reports in the literature of low doses of penicillin used in skin tests or following inhalation eliciting systemic reactions (Bierlein, 1956; Reisman and Arbesman, 1968), but clearly the routes of administration are inappropriate to an analysis of oral challenge doses. There are, however,

some reports which can be used to address this question. The best evidence is provided by a patient investigated by Borrie and Barrett (1961). Severe allergic reactions developed during an attempt at oral desensitization with penicillin within a dose range of 3–9 µg. Wicher, Reisman and Arbesman (1969) and Wicher and Reisman (1980) report a well-investigated patient who responded systemically to penicillin in milk and in a soft drink. The eliciting doses were estimated to be of the order of 1359 µg in the milk and 16–34 µg in the soft drink. In a case recorded by Vickers, Bagratuni and Alexander (1958), the eliciting dose may have been of the order of 2400 µg. The difficulty in this type of analysis is that the minimum challenge cannot be assessed and as the assays for penicillin were done some days after the event, actual penicillin levels may have been higher than the assay figures suggest. However, it is possible to get some insight into minimal doses from analyses of this kind.

Tscheuschner (1972) estimated that less than 6 µg penicillin must have been responsible for eliciting a reaction in a patient who had an allergic reaction while eating pork contaminated with penicillin, although in nine well-documented penicillin-allergic patients investigated by Lindemayr *et al.* (1981), 3.6–6 µg penicillin were tolerated when ingested in raw pork. Clearly, there are limits to the information which can be obtained from data of this kind and direct challenge experiments (Lindemayr *et al.*, 1981) have inherent dangers. To overcome this problem, Siegel (1959) used the Prausnitz-Küstner (PK) reaction, in which serum from allergic patients is injected intradermally into the skin of normal recipients. After a latent period the recipient is given the allergen, in this case penicillin, by an appropriate route. Siegel (1959) investigated, by this technique, a number of sera taken from different patients. Serum from one patient responded to an oral penicillin dose of 30 µg with a clear positive response, including whealing, and 24 µg was sufficient to give an erythematous reaction. Assuming that the sensitized patient is, at minimum, 100 times more responsive than the recipient in a PK reaction, Siegel then argues that the threshold dose to challenge a sensitized person would be 0.24 µg. This figure, 0.24 µg or 0.4 unit, has been widely quoted in the literature. It is important, therefore, to understand that it is an extrapolated figure and one significantly lower than those calculated from patients responding to penicillin ingestion. This is particularly relevant to the question of penicillin residues in milk. An individual drinking 250 ml milk containing the accepted level of 0.01 µg/ml could ingest 2.5 µg penicillin, a low figure relative to most of the data on challenge doses.

However, it is clear from the data available that penicillin allergic patients could respond to low levels of penicillin by ingestion, although they do so very rarely if the published literature is an adequate guide. The next section assesses the clinical evidence that adverse reactions in allergic patients have been induced by such low dose exposures to penicillin in milk.

Clinical evidence

As long ago as 1948 it was known that milk from penicillin-treated cows could inhibit cheese-starter cultures. Over the next decade, understanding of the mechanisms involved in the development of penicillin allergy increased and it was not unreasonable that the public health significance of the presence

of penicillin allergens in milk should be questioned. Milk and milk products were regarded as but one source of covert exposure to penicillin, and attention was drawn to residual penicillin in sterilized syringes and from penicillin-producing fungi in the environment (Coleman and Siegel, 1955; Siegel, 1959). Welch (1957) expressed this concern in a publication which recorded that the Commissioners of the Food and Drugs Administration of the USA had authorized the setting up of a Medical Advisory Panel to consider public health problems arising from the presence of antibiotics in marketed milk. The panel took the view that, of the antibiotic residues that might be present in milk, only the case of penicillin required consideration. However, Welch stated at that time that 'We do not have a single proved case of a reaction following the ingestion of fluid milk known to contain penicillin' and he emphasized that the problem of contamination of milk with antibiotics was a small one compared with other food safety problems, including those created by the use of preservatives, colouring agents, stabilizers and flavours.

Eighteen years later, Olson and Sanders (1975), also of the FDA, wrote: 'The problem appears to be of such magnitude that would seem to require the co-ordinated effort of all producer-cooperative organizations to bring the problem under control—and the sooner the better for all concerned.'

The literature has been searched diligently to find the evidence which would support such a dramatic change of view. Two types of hazard or clinical situation were sought. In the first, the published literature was reviewed to establish whether any case histories presented could be interpreted as supportive of the view that residual penicillin in milk could induce sensitization in man *de novo*. In the second, the question was asked whether there were documented cases of the ability of residual penicillin in milk to elicit reactions of an allergic nature in patients with a clinical history of penicillin allergy.

The first is readily answered. There are a few anecdotal cases quoted in the literature (Mauranges, 1972; Cany, 1977), but none is well documented and in spite of the theoretical risk of ingesting low concentrations of an allergen over an extended period referred to earlier, the consensus of those with an interest in this topic is that primary sensitization is a negligible risk.

Before analysis of the second question, that of allergic reactions in penicillin-allergic individuals, it is important to establish the criteria on which the evidence is based. *Table 38.3* lists criteria in an order which gives increasing confidence in the accuracy of the diagnosis. *Table 38.4* summarizes the clinical cases documented in the literature. Against each case is noted the criteria used.

Several points may be made in discussion of these findings. It is clear that

Table 38.3 DIAGNOSTIC CRITERIA USED IN INVESTIGATIONS OF PENICILLIN ALLERGY INDUCED BY MILK

A	Clinical history of adverse reaction to milk ingestion in penicillin-allergic patient
B	Clinical evidence of improvement in condition on milk avoidance
C	Clinical evidence that milk known to be free of penicillin, or penicillinase treated milk, can be tolerated
D	Demonstration of penicillin residues in milk sample which precipitated adverse reaction
E	Clinical evidence of the prophylactic or therapeutic value of penicillinase
F	Immunological evidence to support diagnosis of penicillin allergy and/or lack of sensitivity to milk proteins

there are very few documented cases, a conclusion reached also by Becker (1976) and Adkinson (1980). It could be argued that clinical reactions do occur due to penicillin residues in milk, but are either misdiagnosed or individuals, recognizing that their symptoms are associated with ingestion of milk, act on their own initiative and simply avoid milk and milk products. A recent survey (Harris, 1982) is relevant to this issue. It was shown that, of a survey sample of 8749 people in the UK, 1.5% avoided milk because they perceived it to do them some harm. This group would be expected to include those with cows' milk allergy, lactose intolerance and other syndromes. It is possible that the group also includes some who are allergic to penicillin residues in milk and although their perception of the precipitating cause of their symptoms is correct, the precise aetiology is not apparent to them. However, in view of the low incidence of milk avoidance in the population, based on this survey, and the probable dominance of cows' milk allergy and intolerance in determining avoidance, it is unlikely that there exists in the UK a significant percentage of individuals whose penicillin allergy precipitated by penicillins in milk has gone undiagnosed.

Analysis of data given in *Table 38.4* shows that even the cases that are recorded cannot be said to provide unequivocal evidence of the hazard of penicillin in milk. The diagnostic criteria used in most cases are inadequate in one respect or another. In very few cases was the presence of penicillin shown in the offending milk sample, and only rarely was there sufficient diagnostic evidence of either penicillin allergy or of lack of milk intolerance.

The use of penicillinase is of interest. Penicillinases (β -lactamases) are enzymes which hydrolyse penicillins to penicilloic acids by opening the β -lactam ring. A penicillinase (neutrapen) was first used in this context by Becker (1956) and evaluated extensively by Zimmerman (1957-58). It is no longer used in most countries of the world as therapy in penicillin allergy, partly because of dangers of sensitization and partly because current immunological theory indicates that breakdown products of penicillins might play a role in eliciting penicillin allergy. If penicillinase therapy were responsible, in the cases listed, for the benefit obtained in allergic patients treated with it, it would be justifiable to conclude, first, that penicilloic acid and its further degradation products (*Figure 38.2*) play no part in penicillin allergy induced by penicillin in milk, and, second, that penicilloylated bovine protein conjugates which are unaffected by penicillinase are equally blameless.

Overall, therefore, although it is established that penicillin in milk can elicit reactions in some individuals, the clinical and scientific validity of many of the case reports needs to be evaluated critically in the light of current knowledge and techniques. There seems little in the literature to support the view that there is a problem of significant magnitude and the dramatic view expressed by Olson and Sanders in 1975 seems unwarranted.

Boonk and Van Ketel (1980; 1982) have reported a study which may have some indirect bearing on the question of the significance of penicillin in milk. In an investigation of 252 patients with chronic, recurrent urticaria, 70, or 27.8%, showed either patch test reactions or intradermal responses to penicillin derivatives. Thirty out of 52 of these patients responded clinically to a diet free of milk or milk products, whereas only 2 out of a group of 40 patients with chronic urticaria and negative skin tests responded favourably to this diet restriction. This study is of interest, but cannot be used to support the

Table 38.4 SUMMARY OF REPORTED CASES OF PENICILLIN-SPECIFIC ALLERGIC REACTIONS ELICITED BY MILK

<i>Reference</i>	<i>No. of cases</i>	<i>Clinical response</i>	<i>Criteria (see Table 38.3)</i>	<i>Comments</i>
Erskine (1958) in Mauranges (1972)	1	Dermatitis	A, D	No further details available. Original reference not examined
Vickers, Bagratuni and Alexander (1958)	1.	Contact dermatitis	A, C	Positive patch test to penicillin. No clinical history. No evidence of penicillin in offending milk sample. Intramuscular desensitization claimed to be successful
	2	1. Rash, lymph node enlargement; joint swelling	None	Positive patch test to penicillin, but no clinical history of penicillin allergy. Circumstantial evidence only
Zimmerman (1957-8; 1959)	4	Chronic urticaria, hives	A, C, E	Importance of dairy products; Roquefort cheese stressed. See text for interpretation of criterion E. No evidence (other than indirect through action of penicillinase) of penicillin in these products
Siegel (1959)	1	Generalized immediate hypersensitivity	A, F	A highly sensitive penicillin allergic through IgE mechanisms, but evidence for involvement of milk anecdotal. Criteria C and D not fulfilled
Borrie and Barratt (1961)	1	Dermatitis	A, B, C, D, F	Best documented case, but diagnosis only confirmed by challenge tests. PK test performed but negative
Smythe (1969)	1	Dermatitis, angio-oedema. Recurrence of undulant fever	B	No evidence given of basis of diagnosis of penicillin allergy. No evidence of penicillin in milk consumed
Wicher, Reisman and Arbesman (1969)	1	Pruritis, rash, headache	A, D, F	Well-documented penicillin allergic. Immunology and skin tests indicate IgE-mediated reaction
Cany (1977)	3	Urticaria	A, B, F	Patients said to be sensitive also to pork, veal, chicken. No evidence that penicillin present in offending milk or meat

view that penicillins in milk are of clinical significance. No evidence was presented that there was any penicillin in the milk consumed by these responding patients and sensitivity to milk proteins was not ruled out. The prevalence of positive skin test reactions to penicillin was remarkable and the significance of the results must be questioned in the context of the fact that in only 7 patients out of 99 questioned was there any evidence of a clinical history of penicillin allergy. It is possible that mast cell fragility might have been responsible for this high frequency of skin test reactions in this particular group of patients. Chronic urticaria is a heterogeneous condition of mixed aetiology and the severity of the disease is subject to fluctuations.

Firm conclusions cannot yet be drawn on these data, but the role of penicillin allergy in chronic urticaria bears further investigation whether related to milk as a source of penicillin allergens or not. Penicillin in therapeutic use is possibly the commonest cause of drug-induced acute urticaria, but no evidence is available on which to base a mechanism of action for any role penicillin may have in chronic urticaria. Hidden sources of penicillin are usually indicted, but no evidence has been presented (Jillson and Porter, 1965; Warin and Smith, 1976).

The data of Zimmerman (1957-58) on the use of penicillinase in the treatment of penicillin allergic patients with chronic urticaria suggest that the presence of the penicillin molecule with an intact β -lactam ring is necessary for the prolongation of the urticarial lesion. Thus, when penicillinase was used in therapy the episodes of urticaria were aborted. It might be concluded that this implies that if chronic urticaria can sometimes be due to covert penicillin in milk, then either one has to postulate continuous or at least frequent intermittent exposure to intact penicillin or consider an alternative source of persistent allergen.

In a different context, i.e. following systemic treatment of pigs with benzylpenicillin, Wal (1980) has shown that penicilloylation of porcine serum albumin can occur and that these modified albumins are eliminated by two routes. Rapid elimination follows the interaction of conjugate with antibody to the penicilloyl group, but Wal claims that some penicilloyl groups may be masked inside the tertiary structure of the protein and are thus unavailable to antibody interaction. Normal catalytic breakdown of these proteins would, however, unmask these groups, exposing them to antibody and giving rise to a persistent allergen which could account for late and chronic allergic reactions. Further investigation of this phenomenon is warranted. However, it does not explain the apparent efficacy of penicillinase in the treatment of penicillin allergy elicited by milk, as this enzyme has no effect on penicilloylated proteins.

The work also opens to question the site of penicilloylation of serum albumin and this requires further study as it has implications beyond the narrow issue of penicillin residues in milk.

Discussion

It has proved difficult to quantify the public health significance of penicillin residues in milk. Clarification of the mechanisms involved in penicillin allergy has led to it being regarded as the model system against which other drug-

induced allergic syndromes are measured. There are advantages and disadvantages in this honoured position. Research on the nature of penicillin allergens, including polymers and macromolecular impurities, has led to more attention being paid to the purity and stability of penicillins and this, coupled with the greater use of oral penicillins, has resulted in a lower incidence of clinical reactions. On the other hand, because the propensity of penicillin to cause allergic reactions is so well known, there is a tendency to over-diagnose, and an adverse reaction of an allergic type in a patient receiving a penicillin will thus, on probability grounds alone, be diagnosed as penicillin allergy, without due attention being paid to alternative diagnoses such as pseudo-allergic reactions.

Similar considerations apply in relation to penicillin in milk. There is no doubt that mechanisms exist whereby penicillin-derived allergens might be both immunogenic and allergenic and the nature of the allergens can be, at least in part, defined. There is equally no doubt that a few individuals are so hypersensitive to the penicillin group of antibiotics that they respond adversely to very low challenge doses administered by a variety of routes. These data, however, lead only to the conclusion that penicillins in milk could represent an important covert source of penicillin allergens hazardous to allergic people. From the literature reviewed in this paper, it is clear that this must be regarded as a possibility. On the other hand, the probability that, at currently accepted levels, penicillin residues represent a hazard, is low. Clinical evidence supports this view, in that in spite of intense interest in the topic over many years and costly regulatory involvement, only a very small number of individuals are documented as having had allergic difficulties.

The question that must now be addressed is whether it is justified, in response to ever-increasing assay sensitivity, to aim to reduce still further penicillin residues in milk. This course of action seems unjustified, a view expressed also by Becker (1976). It may be that there will always be one or two individuals of such exquisite sensitivity that they will respond to sub-microgram concentrations of penicillin. Public health regulations, however, cannot be expected to accommodate such individuals, who will learn to avoid foodstuffs which seem to do them harm in the same way that food-allergic subjects do. The cost-benefit equation should dominate public health concerns and no immunological benefits are likely to accrue from further lowering of acceptable levels of penicillin in milk.

Moreover, there would seem to be no justification in introducing an assay for the presence of penicilloylated proteins in milk. The evidence presented in this chapter fails to establish a significant role for them in penicillin allergy, and the kinetics of their disappearance from milk parallels that of penicillin itself. In the experimental work quoted (Wal and Bories, 1975) such penicilloylated proteins had reached undetectable levels at a time after therapy at which there was still 0.06 p.p.m. penicillin, a level above that now acceptable. The simpler assay for penicillin in milk, compared with radioimmunoassay for penicilloylated proteins, would seem therefore to offer an adequate safeguard of the quality of milk.

Finally, this chapter has been addressed to the question posed in its title. The significance of the presence of other antibiotics which might be found in milk as a consequence of treatment of bovine mastitis has not been considered. It is probable that most of the comments made apply equally to the cephalo-

sporins, which, as β -lactam antibiotics, are capable of reactivity similar to that of the penicillins.

The role of tetracyclines and aminoglycosides in inducing allergic reactions has been reviewed (Dewdney, 1977). These antibiotics can participate in immunologically mediated reactions, but it is unlikely that their presence in milk could, as a consequence, be a clinical hazard.

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ANTIBIOTICS IN ANIMALS AND FOOD PROCESSING: ARE THEY HUMAN NUTRITIONAL HAZARDS?

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Introduction

One of the greatest benefits to the human race has been the discovery in nature, or recognition by scientists, of various chemotherapeutic substances (including antibiotics) and the subsequent ability to synthesize and manufacture these substances for controlled use in the human and animal populations. Antibiotics are used in commercial animal production for therapy, prophylaxis or enhanced growth, to maximize the economic returns from individual animals or herds. Other chemical additives with 'antibiotic action' are used against moulds and yeasts to preserve grain and silage for animal feeding. In the human food industry, additives are needed to extend the range of foods and to supplement methods of food handling, packaging and processing. Examples of these are:

- (1) Antioxidants to protect nutrients, colour and flavour.
- (2) Preservatives to retard spoilage.
- (3) Flow agents to prevent lumping and caking.
- (4) Acidulants, flavours, colours, sweeteners, to improve aesthetic appeal.

The use of additives (including antibiotics in their more restricted sense) in both the human and animal food industries is now controlled by law in the UK and elsewhere. Confining attention more specifically to the UK, it can be seen that since the early 1950s until the present moment, several Acts, with subsidiary Regulations, have been passed governing what can be in, or used for, the processing of food. However, limiting attention to UK legislation should not blind us to the necessity to be conscious of what is happening in other countries, since so much imported food is now available to us on supermarket shelves.

To date, the main legislation affecting the inclusion of additives such as antibiotics has been:

- (1) Agriculture (Poisonous Substances) Act (1952).
- (2) Food and Drugs Act (1955) and subsequent Regulations.
- (3) Medicines Act (1968).
- (4) Feed and Fertilisers Act (1973).
- (5) Preservatives in Food, Regulations (1975).

As a result of the Agriculture (Poisonous Substances) Act of 1952, an advisory committee on poisonous substances used in agriculture and food storage was set up, and in 1962 a joint committee of the medical and agricultural research councils was founded, to act as watch-dog and co-ordinating committees. In case some people think we are suffering from too much legislation in this area, perhaps we should remember that food hygiene laws were in operation as far back as 1400 B.C. Biblical scholars may recall the Mosaic Law, written about 1451 B.C., on the eating of poisonous fish: 'Whatsoever hath not fins and scales ye may not eat; it is unclean for you' (*Deuteronomy*, 14, 9-10).

Now, turning our attention more specifically to antibiotics (*in sensu stricto*) usage in food processing, we see that in the 1940s and 1950s antibiotics, for example tetracyclines and chloromycetin (chloramphenicol), were used in the packaging and storage of chicken, fish, fruit and vegetables in this country. They did increase the shelf-life of some foods, but current legislation forbids their present use for these purposes. Only nisin is licensed for use in food processing in cheese, clotted cream and canned food. The Therapeutic Substances Regulations (1958) also permits the use of streptomycin and oxytetracycline for plant disease control. However, permission to include, 'willy-nilly', antimicrobial substances in food processing could in fact lead to the development of slipshod methods and approaches to hygiene. An example of this would—in reverse—be the reluctance of some EC Member States to permit the inclusion of high levels of free chlorine in water of poultry processing plants to control possible salmonella contamination at plants, instead of at source farms.

At this stage, therefore one of the questions posed by the title of this chapter, namely, 'Antibiotics in . . . Food Processing: Are They Human Nutritional Hazards?' could be answered 'no' in UK-produced foods, since legislation precludes their use and the safety—in use—of nisin has been previously considered.

The second question posed is 'Antibiotics in Animals . . . : Are They Human Nutritional Hazards?' It was decided to avoid—as far as it could be foreseen—any such ground as might be covered by other chapters.

The hazards usually cited as potential, or potent, danger areas for the human population when antibiotics are used in animal husbandry are:

- (1) The emergence of resistant strains of bacteria in animals and the passage of these or their resistant factors via the food chain from animals to man.
- (2) The production of harmful effects from direct toxicity or from allergic reactions in persons who had previously been sensitized to them.

If the latter hypothesis is accepted, how could it arise? One way is via residues in the meat we consume from our local butcher, supermarket or deep freeze supplier. Remember, the occurrence of antibiotic residues in animal products is a function of: (a) the nature of the antibiotic and its formulation; (b) the method or mode of application; (c) the treatment level; and of most importance (d) adherence to withdrawal periods.

The most important of these four factors, from the tissue residue point of view, is strict adherence to the prescribed withdrawal period of the drug before the animal is sent for slaughter for human consumption. All drug companies now making application for licences for new products must furnish

withdrawal data to support their submissions. However, in spite of legislation, it is possible on occasion for animals to be sent for slaughter with residues of antibiotics in their tissues. In the Stormont laboratory, residues have been detected in meat, randomly sampled at slaughter-houses. It is an infrequent occurrence, but it can happen. In order, therefore, to help minimize any potential risk to consumers of meat containing such residues, it was considered just what effect cooking or prolonged cold storage could have on meat containing antibiotic residues. Meat inspection and hygiene is under veterinary control by the Department of Agriculture in Northern Ireland, and the Veterinary Research Laboratories at Stormont are called upon from time to time to advise on problems such as residues in emergency slaughtered animals, and to carry out residue testing on carcass meat being sent, for example, to Germany. The cooking and cold storage aspect of this work, should be of interest and will not have been covered in other contributions.

An outline of one such investigation is as follows. Five antibiotics—ampicillin, chloramphenicol, oxytetracycline, streptomycin and sulphadimidine, which are used fairly widely in animal treatment, both legally and illegally—were selected. They have varying modes of action and pharmacodynamics. These were injected, intramuscularly, into bovines using 2 per antibiotic. The levels (therapeutic) used per kilogram bodyweight were 7 mg/kg (ampicillin), 4.5 mg/kg (chloramphenicol), 5 mg/kg (oxytetracycline), 2 mg/kg (streptomycin) and 14 mg/kg (sulphadimidine). They were injected once daily on 3 consecutive days and, within 2 h after the third injection, were euthanased. This regime ensured tissue residues. Each carcass was skinned, eviscerated, split into two sides and left to hang at $+4^{\circ}\text{C}$ for 5–7 days to simulate normal trade practice. Immediately after slaughter, samples were tested for the presence of the antibiotic administered. At the end of the hanging period, samples were again tested. The minimum amounts of the antibiotic detected in the tissues of the test animals (A and B) were as shown in *Table 39.1*.

Table 39.1 MINIMUM AMOUNTS OF ANTIBIOTICS DETECTED IN TEST ANIMALS

<i>Antibiotic</i>	<i>Test animals</i>	
	A ($\mu\text{g/g}$)	B ($\mu\text{g/g}$)
Ampicillin	0.6	0.8
Chloramphenicol	1.6	1.5
Oxytetracycline	0.5	0.6
Streptomycin	6.4	—
Sulphadimidine	8	8

After the hanging period, one sirloin roast and three steaks were cut from each carcass side. Roasting and grilling were done on an ordinary domestic cooker. Thermocouples were inserted into the centre and sub-surface of the roasts and into the thickest part of each steak during the cooking, and temperatures were monitored at these sites on a continuous flat bed recorder. In addition to cooking, individual samples of muscle tissue were prepared from the remainder of the hind-quarters of each carcass, and put under refrigeration at -20°C . At different time intervals individual samples were retrieved, allowed to thaw at room temperature, and assayed. This aspect of

the investigation continued for up to 80 weeks. After each cooking session, samples of each roast were taken from (a) the outer surface; (b) about mid-depth; and (c) at the centre.

Results

The results of the microbiological assays of each antibiotic were recorded as zones of inhibition (mm). However, in order to make the results more meaningful and readily intelligible, the results were expressed as the reduction in annular zone size diameter after cooking or storage as a percentage of pre-cooking or pre-storage annular zone diameter. While percentage reduction of annular zone size diameter is used to indicate reduction in biological activity, it should be remembered that there is not a direct correlation between the two.

The results of the experiment indicated that there was on average, in all the rare steaks, less than 10% reduction in the annular diameter size, the range being from no reduction (chloramphenicol) to 15.8% (oxytetracycline). For ampicillin, chloramphenicol and oxytetracycline, the reduction in the medium and well-done steaks and roasts varied considerably and appeared to depend on (a) the temperature attained during cooking and (b) the duration of cooking. For example, no residue of ampicillin was detectable in the surface meat of the roasts from one animal when the maximum temperature was 93°C or 96°C, the total cooking times being 180 and 210 min, respectively. However, when the temperatures recorded in the medium steaks were in the 50s Celsius, there was only a reduction of 2.3–13.3% in zone diameters.

The sulphadimidine residues in any of the steaks or roasts were affected either minimally or not at all by either temperature rise or the duration of exposure. No cooking experiments were carried out on the muscle tissue from the streptomycin-injected animals, as previous experience with experimental animals had confirmed the difficulty of recovering biologically active streptomycin from muscle tissue. In all, 16 roasts and 48 steaks were assayed.

Some idea of the temperature patterns recorded during the experiment may be seen by typical patterns, together with the mean of the recorded temperature maxima (*Figures 39.1 and 39.2*).

During the cold storage part of the experiment it was not feasible, due to deterioration, to keep any tissues at +4°C longer than 6 weeks. The effects of cold storage at this temperature on the residues of the different antibiotics was very variable. Whereas storage at +4°C resulted in the reduction of ampicillin and chloramphenicol to low levels, minimal, if any, effects were observed in oxytetracycline, streptomycin and sulphadimidine residues at this temperature. At –20°C there was practically no reduction in the residue levels of chloramphenicol, oxytetracycline and sulphadimidine in muscle, and reductions of 19.5–38.4% of ampicillin residues. The effect of cold storage on the residues in kidney and liver tissues was also slight, except for ampicillin where storage over long periods did decrease the levels detectable in the liver.

To recapitulate, the investigations showed that active antibiotic residues could be detected in animal tissues after roasting, grilling and prolonged cold storage. Fuller details of this investigation have been published elsewhere (O'Brien, Campbell and Conaghan, 1981).

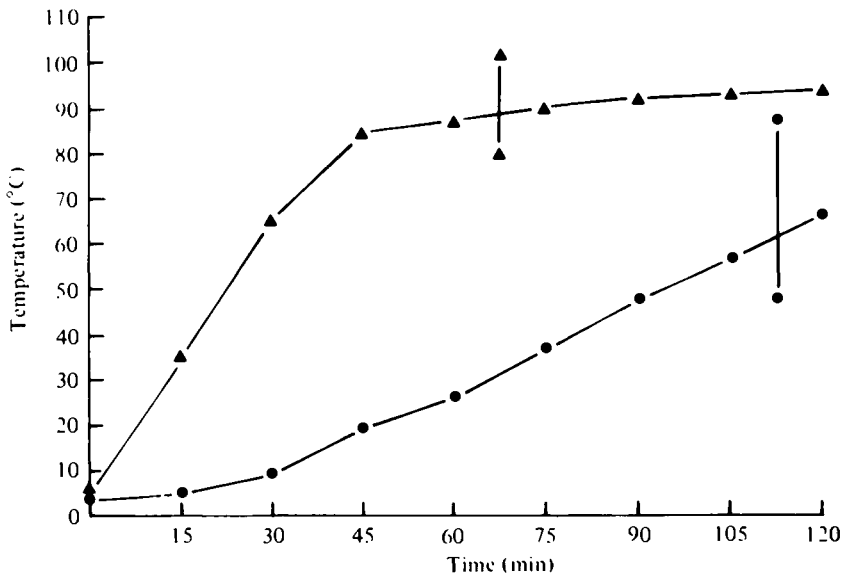


Figure 39.1 Temperature increases during cooking of roasts from sulphadimidine-injected animal (B). Bars indicate the range of maxima temperatures of all 16 roasts in the whole experiment: ●—●, roast (centre); ▲—▲, roast (outside)

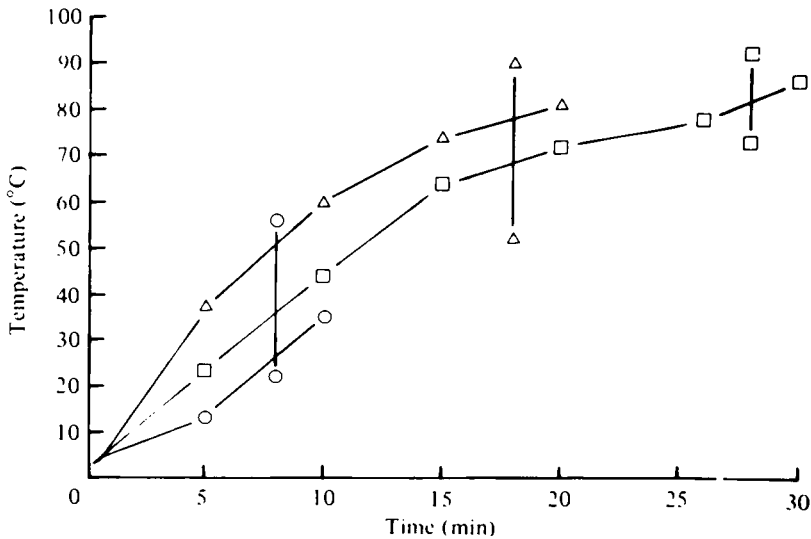


Figure 39.2 Temperature increase during cooking of steaks from sulphadimidine-injected animal (A). Bars indicate the range of maxima temperatures of the 16 steaks used for each grilling regime: ○—○, steak 'rare'; △—△, steak 'medium'; □—□, steak 'well done'

However, to allay any disquiet, it should be mentioned that most antibiotics are administered to farm stock in the feed or water. This route of medication has many advantages. First, it gets over the chore of the individual handling of the particular animal and, with the formulations used, absorption of the drugs across the gut wall can be controlled to a greater extent than when administered parenterally.

A recent check carried out at the Veterinary Research Laboratories in Stormont, using poultry broilers with the growth promoters/coccidiostats, avoparcin, monensin sodium, virginiamycin and zinc bacitracin, showed that even after 2 weeks' feeding of these substances and without any withdrawal period, no residues of these substances could be detected in muscle, liver or kidney of the birds. The levels used in the broiler rations are shown in *Table 39.2*.

Table 39.2 LEVELS OF GROWTH PROMOTERS/COCCIDIOSTATS USED IN BROILER RATIONS

<i>Substance</i>	<i>Level in feed (mg/kg)</i>
Avoparcin	10
Monensin sodium	100
Virginiamycin	10
Zinc bacitracin	2100 (iu)

Since chlortetracycline can be used frequently in farm stock (including broilers), it was fed at the level of 200 mg/kg of feed to another batch of broilers. Muscle, liver and kidney were again assayed, without a withdrawal period, when the broilers had been on the medicated diet for 2 weeks. Residues were found in all those tissues. However, if the medication was withdrawn 5 days prior to slaughter, then it was not possible to detect any residues in muscle, liver and kidney. The levels of sensitivity of the assay system are given in *Table 39.3*.

Table 39.3 SENSITIVITY LEVELS OF THE ASSAY SYSTEM

<i>Antibiotic</i>	<i>Level of sensitivity (µg/g)</i>
Avoparcin	0.03
Chlortetracycline	0.001
Monensin sodium	0.03
Virginiamycin	0.007
Zinc bacitracin	0.003 (iu)

Conclusions

To sum up and to attempt to answer the questions inherent in the title of this chapter it is suggested:

- (1) Since no antibiotics other than nisin are permitted to be incorporated

directly in human food processing, no health hazard should arise from such incorporation.

- (2) Should for one reason or another antibiotic residues be present in edible tissues, cooking and/or cold storage will act as minimal safeguards in destroying these.
- (3) When antibiotics are being used in animal husbandry, either prophylactically or therapeutically, orally or parenterally, then the withdrawal periods recommended by the manufacturers should, before the animals or their products are sent for slaughter or processing, be strictly adhered to. By observing these recommendations no residues in tissue should ensue.

Reference

- O'BRIEN, J.J., CAMPBELL, N. and CONAGHAN, T.(1981). Effect of cooking and cold storage on biologically active antibiotic residues in meat. *Journal of Hygiene*, **87**, 511-523

HUMAN AND ANIMAL CONSUMPTION OF ANTIBIOTICS AND CHEMOTHERAPEUTIC DRUGS IN SWEDEN DURING 1980

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Introduction

The worldwide use of antibiotic and chemotherapeutic drugs* is often questioned (WHO, 1978; 1981; Conference, 1981). Special attention has been paid to such use in animals due to its possible threat to antibiotic therapy in humans. In order to achieve a basis for further studies, this chapter presents a study of the total consumption of antibacterial drugs in man and animals in Sweden.

Materials and methods

This study includes all antibiotic and chemotherapeutic drugs used for systemic treatment as well as for local and external use. For each drug, the weight of active substance was calculated. The sales of all the mentioned drugs available in Sweden during 1980 were kindly provided by the National Corporation of Swedish Pharmacies, National Agricultural Marketing Board, and ADA Company, Gothenburg.

The number of humans and animals in Sweden during 1980 was calculated as the sum of those individuals dead or slaughtered during the period and the number of those living at the end of the year. The number of food- and fur-producing animals as well as humans was obtained from the 1980 Statistical Abstract of Sweden, the 1980 Yearbook of Agricultural Statistics, the National Board of Agriculture, the National Veterinary Institute and the Swedish National Food Administration. The mean bodyweight of animals was obtained from the Swedish Farmers' Meat Marketing Organisation and of humans from the Swedish Institute for Textile Research. The bodyweights were transferred to metabolic weights (metabolic weight = bodyweight^{0.73}) according to Brody (1945).

* In this chapter, both antibiotic and chemotherapeutic drugs are referred to as antibiotics.

Results and discussion

The total human and animal consumption of antibiotics during 1980, expressed as weight of active substance, was 70.7 and 67.8 tons*. The corresponding data for only the antibacterial drugs was 69.4 and 43.3 tons. Of the latter figure, 14.6 tons are growth-promoting feed additives and thus 28.7 tons are used for treatment of antibacterial infections in animals compared to 69.4 tons in humans. As can be seen in *Table 40.1*, the use of the various antibiotics formed different patterns for human and veterinary medicine. For

Table 40.1 CONSUMPTION OF ANTIBIOTIC AND CHEMOTHERAPEUTIC DRUGS IN SWEDEN DURING 1980

Substance	Drug consumption	
	Human (kg active substance)	Animal (kg active substance)
Sulphonamide	16 033 (22.7%)	6 600 (9.7%)
Penicillin	39 488 (55.9%)	5 317 (7.8%)
Tetracycline	2 216 (3.1%)	9 819 (14.5%)
Aminoglycosides	126 (0.2%)	5 274 (7.9%)
Macrolides	3 913 (5.5%)	603 (0.9%)
Cephalosporins	1 137 (1.6%)	—
Chloramphenicol	149 (0.2%)	47 (0.1%)
Trimethoprim	1 502 (2.1%)	134 (0.2%)
Other antibacterial substances and substances against special infections	4 793 (6.8%)	862 (1.3%)
Antimycotic drugs	1 111 (1.6%)	135 (0.2%)
Antiviral drugs	<1	—
Antiparasitic drugs	232 (0.3%)	13 434 (19.8%)
Coccidiostatic feed additives	—	10 937 (16.1%)
Growth-promoting feed additives	—	14 630 (21.6%)
Total (tons)	70.7	67.8

example, human consumption was dominated by penicillin (55.9%), whereas this drug only constituted 7.8% of the antibiotics given to animals. Moreover, both in actual number and relatively the use of the antiparasitic drugs tetracycline and aminoglycosides and, of course, of antibiotics used as growth-promoting and coccidiostatic feed additives was larger in animals than in humans. The data of *Table 40.1* are also presented graphically in *Figures 40.1–40.3*. The growth-promoting antibiotics are presented in *Table 40.2*.

Table 40.1 covers antibiotics belonging to about 115 different pharmacological groups. Out of these, 70 are used only by humans and 30 only by animals. The latter figure includes 15 antiparasitic and coccidiostatic drugs and 8 growth-promoting feed additives. Only about 15 of the different pharmacological groups contain drugs used both by humans and animals.

A more detailed study of the use of penicillin is given in *Table 40.3*. It can be seen that nearly all (98.7%) of this use in animals consisted of penicillin sensitive to penicillinase, compared to 76.2% for human use. The latter, in addition, includes 9.4 tons (23.8%) of penicillins resistant to penicillinase and penicillin with a broad activity, compared to 69 kg (11.3%) for the corresponding use in animals. It was also found that about 8% of the human use of

* 1 ton = 1.016 05 tonnes.

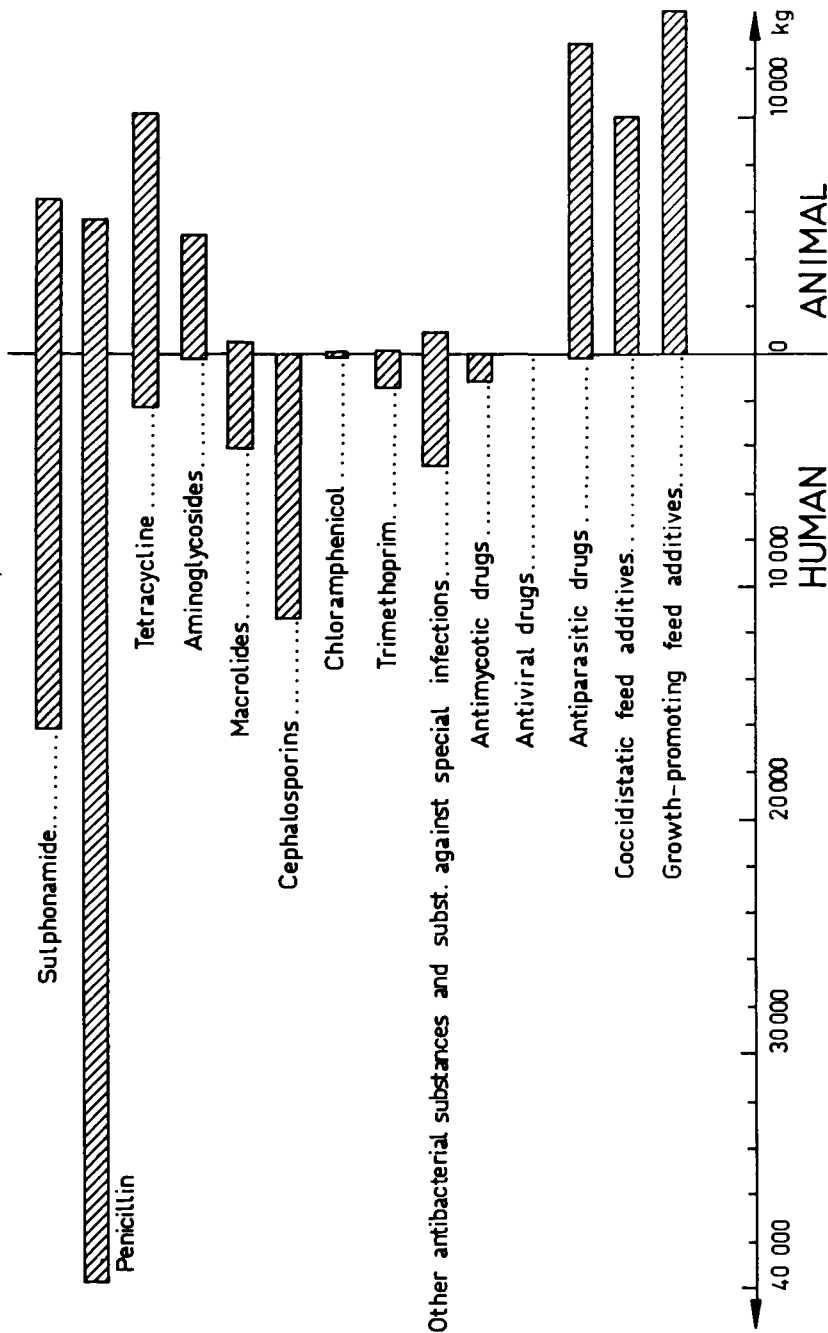


Figure 40.1 Consumption of antibiotic and chemotherapeutic drugs in Sweden during 1980 (kg active substance)

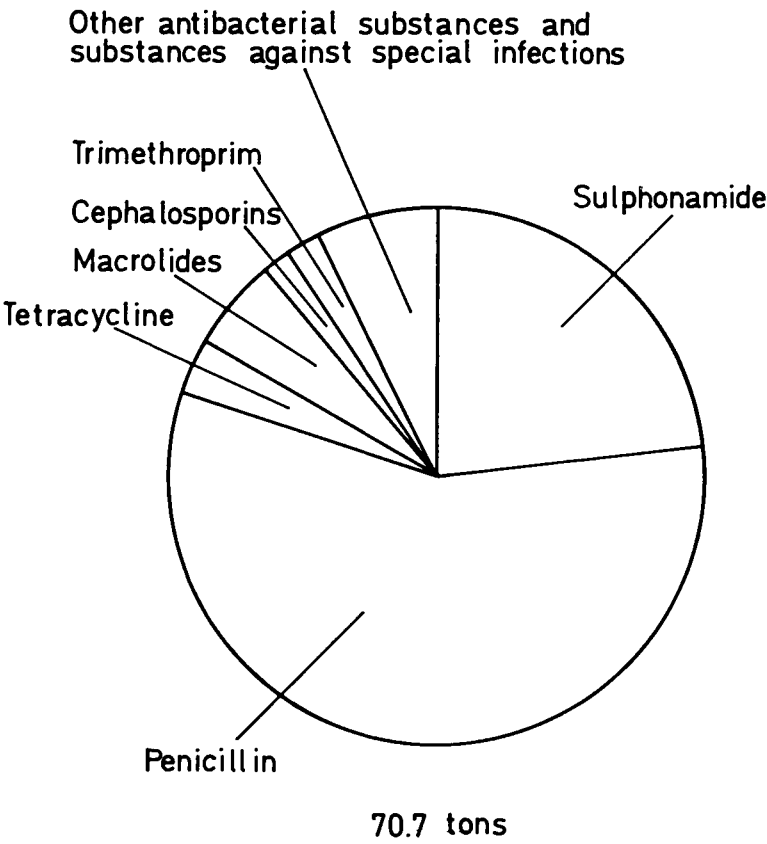


Figure 40.2 Consumption of antibiotic and chemotherapeutic drugs by humans in Sweden during 1980

Table 40.2 CONSUMPTION OF ANTIBIOTIC FEED ADDITIVES IN SWEDEN DURING 1980

<i>Use</i>	<i>Additive (tons active substance)</i>	
Growth-promoting additives (prescription free)	Avoparcin	7.35
	Bacitracin	0.62
	Flavomycin	<0.00
	Nitrovin	0.03
	Oleandomycin	0.12
	Spiramycin	0.07
	Bayonox	4.32
	Mecadox	1.93
	Chlortetracycline ^a	0.19
		14.63
Therapy additives (by prescription)	Chlortetracycline	8.34
	Oxytetracycline	0.74
	Spiramycin	0.12
		9.20

^a For use on mink farms by prescription.

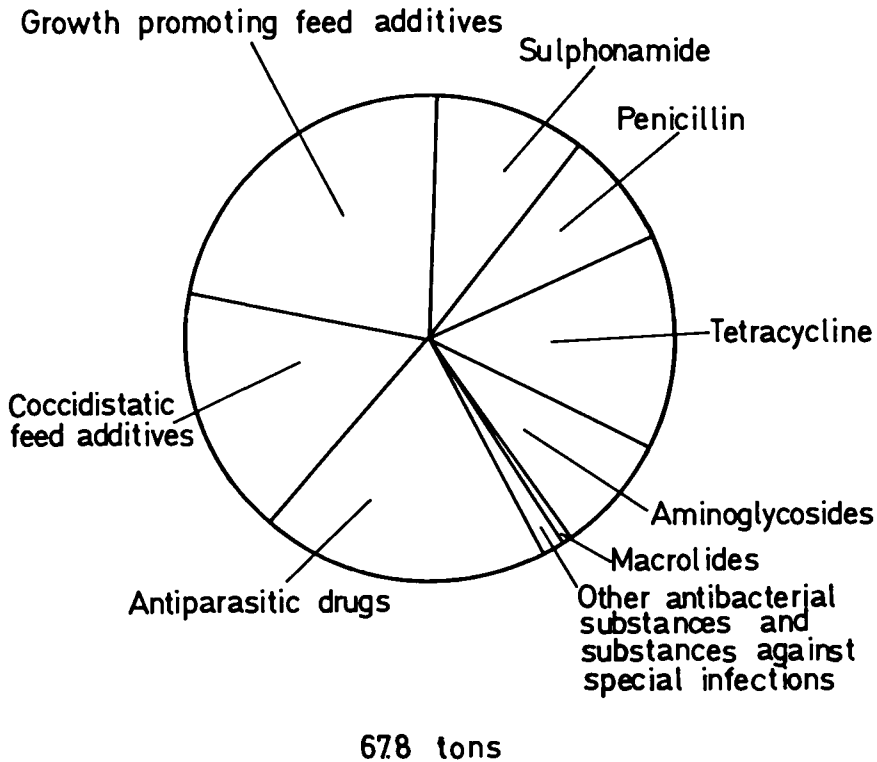


Figure 40.3 Consumption of antibiotic and chemotherapeutic drugs in Sweden during 1980

Table 40.3 CONSUMPTION OF PENICILLIN IN SWEDEN DURING 1980

<i>Type of penicillin</i>	<i>Penicillin consumption</i>	
	Human (kg active substance)	Animal (kg active substance)
Penicillinase-sensitive (total)	<u>30 083</u> (76.2%)	<u>5 248</u> (98.7%)
benzylpenicillin	988	207
benzylpenicillin procaine	< 1	5 041
phenoxymethyl penicillin	28 447	—
azidocillin	648	—
Penicillinase-resistant (total)	<u>4 162</u> (10.5%)	<u>9</u> (0.2%)
dicloxacillin	1 233	—
cloxacillin	1 589	9
flucloxacillin	1 340	—
Broad-spectrum activity (total)	<u>5 243</u> (13.3%)	<u>60</u> (1.1%)
ampicillin	1 527	60
amoxicillin	2 094	—
pivampicillin	1 415	—
carbenicillin	103	—
bacampicillin	104	—
Total (tons)	39.5	5.3

penicillins was given by injection, compared to over 99% of the corresponding animal use. However, this figure is probably somewhat lower, as many antibiotics for per oral and external use for small animals (dogs, cats) are the same as those used by humans and, therefore, cannot be separated from human use. This is the reason why dogs and cats are excluded from *Table 40.4*, which is the basis for the calculation of the antibiotic consumption in relation to bodyweight presented in *Table 40.5*. This table demonstrates that the consumption of antibacterial drugs per metabolic bodyweight was 6.5 times larger in humans than in animals. The corresponding figure is 4.2 when all antibiotic and chemotherapeutic drugs are included.

Table 40.4 BODYWEIGHT AND NUMBER OF PEOPLE AND FOOD- AND FUR-PRODUCING ANIMALS IN SWEDEN DURING 1979

	<i>Number^a</i> (millions)	<i>Bodyweight</i> (ton ⁵)	<i>Metabolic bodyweight</i> (ton ⁵)
Swine	6.81	5.67	1.67
Cattle	2.59	11.41	3.85
Sheep	0.68	0.30	0.11
Poultry	55.64	0.73	0.72
Horse	0.12	0.50	0.11
Reindeer	0.24	0.15	0.04
Mink	1.77	0.25	0.23
Fox	0.06	0.04	0.02
Chinchilla	0.01	<0.01	<0.01
Fish	600 tons	0.06	0.06
		19.12	6.82
Human	8.39	5.16	1.67

^a Number of individuals at the end of the year, plus those slaughtered or dead during the same year.

Table 40.5 CONSUMPTION OF ANTIBIOTIC AND CHEMOTHERAPEUTIC DRUGS BY HUMANS AND ANIMALS IN RELATION TO BODYWEIGHT IN SWEDEN DURING 1980

	<i>Human</i>	<i>Animal</i>
Consumption of antibacterial drugs, tons of active substance (A_1)	69.4	43.3
Total consumption of antibiotic and chemotherapeutic drugs, tons of active substance (A_2)	70.7	67.8
Bodyweight, ton	0.52	1.9
Metabolic bodyweight, ton (B)	0.17	0.68
A_1/B	408.0 (C_1)	63.7 (D_1)
A_2/B	415.9 (C_2)	99.7 (D_2)
C_1/D_1		6.4
C_2/D_2		4.2

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EVALUATION OF THE EUROPEAN COMMUNITY'S FOUR-PLATE METHOD FOR THE DETECTION OF RESIDUES OF ANTIMICROBIAL DRUGS IN SLAUGHTERED ANIMALS

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Introduction

Antibacterial drugs (antibiotics) are widely used in food-producing animals for therapy, prophylaxis and growth promotion. From a public health point of view these applications can lead to unacceptable residues in food derived from those animals. At present, only antibiotics used as feed additives are subject to European Community (EC) regulation and may be given without interference of a veterinarian. All other applications are subject to veterinary prescription. Several countries have specific legislation requiring meat to be free from residues of antibiotics. The Third Country Directive (1972) of the EC requires that meat imported into the Community shall be free from antibiotic residues and it is expected that a similar Directive will be made on intra-Community trade. For this purpose, Bogaerts and Wolf (1980) published a standardized, sensitive microbial method as a result of the work of an EC scientific working group, consisting of representatives of nine Member States. The European Community test (ECT) is a four-plate agar diffusion test, based on various existing test systems: the German Hemmstoff test (Levetzow and Weise, 1974), a variant of the Dutch *Sarcina lutea* kidney test (Schothorst and Peelen-Knol, 1970) and a plate containing trimethoprim (TMP) in order to increase the sensitivity to sulphonamide residues (Gudding, 1976).

The test is proposed for routine testing of fresh meat samples. Kidneys and livers can also be examined, but interfering factors should be taken into account in these cases, especially if samples are frozen. Residues of the β -lactam antibiotics, tetracyclines, aminoglycosides, macrolides and sulphonamides can be detected with this method at a reasonable level. The test is insensitive for residues of chloramphenicol (Nouws, Schothorst and Ziv, 1979; Nouws, 1981). Most of the detection levels, which have been published, are based on experiments using standard solutions of antibiotics or spiked samples of meat homogenates (*Table 41.1*, second column).

However, a routine test for monitoring residues of antibacterial drugs in animal tissues should not only be sufficiently sensitive and standardized, but also specific and easy to perform. Information on these latter aspects of the test were not sufficiently available in the literature. Therefore, further research

Table 41.1 MAXIMUM PERMISSIBLE LEVELS AS ASSESSED BY THE DUTCH PUBLIC HEALTH COUNCIL AND MINIMAL DETECTABLE LEVELS AS DETERMINED BY THE ECT OF TEN ANTIBACTERIAL DRUGS

<i>Antibacterial drug</i>	<i>Concentration (µg/g) in muscle tissue</i>	
	<i>Drug tolerance + motive^a</i>	<i>Detection level ECT</i>
Penicillin G-Na	0.005 A	0.05
Ampicillin	0.005 A	0.02
Chlortetracycline	0 T	0.1
Oxytetracycline	0 T	0.5
Streptomycin	0.25 M	0.2
Neomycin	0.25 M	0.2
Erythromycin	0.03 M	0.1
Tylosin	0.03 M	0.2
Chloramphenicol	0 T	> 5
Sulphadimidine	1 M	1

Source: from Public Health Council (1980).

^a A, allergenic motives; M, microbiological motives; T, toxicological motives.

was needed and experience was to be acquired on application of the test in routine practices. In this study, the ECT was performed in 13 Meat Inspection Services in The Netherlands. Investigations were made on samples of muscle tissue and kidneys from slaughtered animals. Positive samples were further analysed by means of a high-voltage electrophoretic (HVE) technique, in order to obtain an indication of the identity of the antibacterial substances involved.

Materials and methods

SAMPLING

A total of 2910 samples of muscle tissue (diaphragm muscle) as well as kidneys were taken hygienically from slaughtered animals, most of which were suspected of having been treated with antibiotics. Thirteen Meat Inspection Services in The Netherlands participated. The samples of muscle tissue were quickly deep frozen (-18°C) and the kidneys kept at 4°C until testing on the same day. After sampling, kidneys were also quickly deep frozen. Samples of both muscle tissue and kidneys which were intended for further analysis by HVE were transported in a deep-frozen state to the National Institute of Public Health. Immediately upon arrival the samples were placed in a deep freeze (-20°C) again until further testing.

PREPARATION OF SAMPLES

Samples of muscle tissue were removed from the deep freeze and allowed to thaw until the outer surface could be removed by means of a sterile scalpel. A cylindrical piece of meat was sampled by means of a sterile cork borer (internal diameter 8 mm). The cylindrical core was sliced into discs, 2 mm thick. Slices (2 mm thick) were cut from the kidneys after the surface had been

removed, using a sterile scalpel. Using a sterile cork borer (internal diameter 8 mm), cylindrical pieces were cut from the slices. Sampling from deep-frozen kidneys was performed as described for muscle tissue.

THE ECT

The ECT was performed at the different Meat Inspection Services according to the prescriptions of Bogaerts and Wolf (1980). The main characteristics of the test are summarized in *Table 41.2*. Two discs of both kidneys as well as

Table 41.2 EC FOUR-PLATE METHOD (ECT)

Test strain	<i>Bacillus subtilis</i> BGA	<i>B. subtilis</i> BGA	<i>Micrococcus luteus</i> ATCC 9341	<i>B. subtilis</i> BGA
Medium ^a	S II N	S II N	S II N	S II N + 0.05 p.p.m. TMP
pH	6	8	8	7.2
Inoculum	10 ⁴ /ml	10 ⁴ /ml	10 ⁴ /ml	10 ⁴ /ml
Incubation temp.	30°C	30°C	37°C	30°C
Incubation time	c. 18 h	c. 18 h	c. 18 h	c. 18 h
Test positive if	≥ 2 mm	≥ 2 mm	≥ 2 mm	≥ 2 mm
annular zone size				
Test disc ^b	0.01 iu pen	0.5 µg str	0.5 µg str	0.5 µg sdm
Annular zone size	≥ 6 mm	≥ 8 mm	≥ 6 mm	≥ 7 mm

^a S II N = Standard II Nähragar.

^b pen, penicillin G-Na; str, streptomycin; sdm, sulphadimidine.

muscle tissue were diametrically placed onto each of the four test plates using a sterile forceps.

A positive result after incubation was indicated by the complete inhibition of growth on the surface of one or more of the four plates around both discs of kidney or/and muscle tissue samples, in an annular zone not less than 2 mm wide.

THE HVE

Equipment

The basic equipment was similar to that described by Lightbown and de Rossi (1965). It consisted of a hollow aluminium cooling block (20 cm × 92 cm) and four Perspex buffer boxes (capacity 720 ml each) and platinum wire electrodes wound around shaped glass rods, all enclosed in a Perspex box. The hinging lid of the latter activated safety switches. The cooling block was connected by flexible tubing to a cooling device with a 100 l/h capacity of water of 10°C. The block permitted support to a sheet of glass (20 cm × 92 cm × 0.5 cm) carrying a slab of the electrophoresis-growth agar. Sheets of filter paper (Schleider and Schull) made connection between the agar slab and buffer boxes at each end and between the latter and second boxes containing the electrodes. The electrodes were connected to a power supply capable of 2500 V and stabilized to provide a constant current of 100 mA.

Test organisms

Bacillus subtilis BGA, *Micrococcus luteus* ATCC 9341 and *Escherichia coli* 28 PR 271 were used. The suspensions of the first three test organisms were prepared as described for the ECT (Bogaerts and Wolf, 1980). The suspension of *E. coli* was prepared as described by Nouws (1978).

Media and buffer solutions

The same media were used for electrophoresis as well as growth of the test organisms. The composition of the buffer solutions was identical to the corresponding growth medium, except that agar was omitted. The medium for *B. subtilis* and *M. luteus* was Elmo-agar and for *E. coli* synthetic medium:

<i>Elmo-agar</i>		<i>Synthetic medium</i>	
Peptone	6 g	Asparagine	1 g
Tryptone	4 g	NH ₂ HPO ₄ ·12H ₂ O	6.3 g
Yeast extract powder	3 g	KH ₂ PO ₄	1.0 g
Lab lemco powder	1.5 g	Na-citrate	1.5 g
Dextrose	1 g	MgSO ₄ ·7H ₂ O	0.2 g
Purified agar (Oxoid L28)	7.5 g	Glycerol	22.5 ml
Distilled water to	1 l	Agar (Oxoid no. 1)	6 g
		Distilled water to	1 l

The ingredients were mixed, dissolved at 100°C and sterilized by autoclaving at 121°C for 15 min. The media were cooled to 45°C and the pHs adjusted using 0.1 N-HCl or 0.1 N-NaOH so that, at 25°C, pHs were obtained of 6 and 8 (*B. subtilis*), 8 (*M. luteus*) and 7.2 (*E. coli*). A clean glass plate was placed on a heating levelling platform and on it was sealed, with melted medium, a non-corrosive metal frame (inner dimensions 17 cm × 90 cm × 0.5 cm). The plate was heated to c. 50°C by circulating hot water through the platform. The appropriate microorganisms were added to the media at 45°C to give a concentration of c. 10⁴ colony-forming units/ml and carefully mixed. The 200 ml of the medium-bacterium mixture was poured onto the plate to give a uniform agar depth of 1.3 mm. The agar layer was allowed to solidify quickly by circulating cold water through the levelling platform.

Preparation of samples

Samples were prepared as described for the ECT.

Electrophoresis procedure

After solidification, the metal frame was removed and the plate was placed on the cooling block of the electrophoresis device. The agar layer was connected to the corresponding buffer solutions (450 ml per buffer box) by layers of filter paper (3 sheets/layer) soaked in buffer solution. Six samples and a filter-paper disc containing penicillin (0.005 E) + streptomycin (1 µg) + chloramphenicol (5 µg) + sulphadimidine (0.5 µg) + 0.01 ml of a solution of brilliant black (0.1%) were placed at equal distances from each other on a line in

the middle between both ends of the plate. A potential of c. 2000 V was applied to the electrodes, giving a constant current of 100 mA. After 1 h, the electrophoresis was stopped and the samples and the paper disc were carefully removed. After repeated wetting of the paper bridges with buffer solution, the electrophoresis was continued for different periods of time to allow the brilliant black indicator to reach a distance from the centre of the plate as follows: 30 cm for *B. subtilis* pH 6, 40 cm for *B. subtilis* pH 8 and *M. luteus* pH 8, and 35 cm for *E. coli* pH 7.2. After termination of the electrophoresis, the paper bridges were removed and the agar layer superficially dried with absorbing filter paper. The metal frame was carefully replaced and covered with a metal lid. The lid consisted of two layers. The lower one was perforated and in between the layers a sheet of absorbent paper was placed. The plates were incubated for 18 h at 37°C.

Reading and interpretation of results

After incubation, the plates were examined for zones of inhibition. The shape and the nearest and farthest points of the zones, measured from the mid-application points of the samples, were compared with pictures and reference data of zones of known antibiotic standards and of non-specific inhibitors, allowing identification in most instances. In cases of unidentifiable zones, tests were repeated after application of a small piece of semi-permeable membrane between sample and agar. In this way, large molecular aspecific inhibitory substances were hindered to diffuse into the agar, permitting the small molecular antibiotics to pass freely.

Results

The ECT was applied to samples of kidneys and muscle tissue from 2910 slaughtered animals by 13 regional Meat Inspection Services. Most animals were selected because of being suspected of having been treated with antibiotics. Pigs formed the vast majority (80%) of the animals that were examined. Antibacterial substances were demonstrated to be present in kidney

Table 41.3 COMPARATIVE DETECTION OF ANTIBACTERIAL SUBSTANCES IN KIDNEYS AND MUSCLE TISSUES OF SLAUGHTERED ANIMALS AS DETERMINED BY THE ECT

Animals species	Test results ^a		Totals
	+ / +	+ / -	
Pig	32	97	129
Veal calf	14	47	61
Beef	8	12	20
Horse	2	3	5
Sheep	1	9	10
Totals	57	168	225

^a + / +, kidney and muscle tissue positive; + / -, kidney positive and muscle tissue negative.

samples from 225 animals (Table 41.3). In samples of muscle tissue from 57 of these animals, inhibitory substances were demonstrated as well.

In order to obtain an indication of the identity of the substances, a number of positive specimens were taken at random for further investigation by means of HVE. The presence of antibacterial drugs could be confirmed in about 50% of the 162 positive kidney samples that had further been investigated (Table 41.4). For 40 samples of positive muscle tissue, the confirmation was 37% (Table 41.5). Inhibition by aspecific substances was obtained with 34% of the kidneys and 20% of the muscle tissues. In order to know which plate of the ECT yielded the most reliable results, based on HVE confirmation, the results of kidneys were further analysed (Table 41.4). The confirma-

Table 41.4 COMPARATIVE INVESTIGATION BY HVE OF KIDNEYS HARBOURING ANTIBACTERIAL SUBSTANCES AS DETERMINED BY EACH OF THE FOUR PLATES OF THE ECT

Plate	Total no.	Positive ^a	HVE result		Conf. (%) ^b
			Aspecific	Negative	
<i>Bacillus subtilis</i> , pH 6	75	55	19	1	73
<i>B. subtilis</i> , pH 8	78	62	15	1	79
<i>Micrococcus luteus</i>	55	42	11	2	76
<i>B. subtilis</i> , TMP	112	53	36	23	47
ECT complete	162	82	55	25	51

^a Streptomycin 71/penicillin 19/neomycin 6/tetracycline 1/chloramphenicol 1/sulphadimidine 1.

^b Percentage confirmed.

tions of the first three plates were c. 75% and that of the TMP plate was 47%. Moreover, inhibitions were also mainly derived from the TMP plate. Further analysis of positive results of samples of muscle tissue gave a different picture (Table 41.5). The results of the second plate (*B. subtilis*, pH 8) of the ECT could be confirmed for more than 80% and those of the other plates for 50% or less.

Next, the part played by each of the four plates of the ECT in producing a positive test result (Table 41.6) was studied. The first plate (*B. subtilis*, pH 6) and fourth plate (TMP) were responsible for the greatest number (12 out of 15) of confirmed positive results obtained with samples of muscle tissue. The additional 3 positives were obtained by the effort of using the other 3 extra

Table 41.5 COMPARATIVE INVESTIGATION BY HVE OF MUSCLE TISSUE SAMPLES HARBOURING ANTIBACTERIAL SUBSTANCES AS DETERMINED BY EACH OF THE FOUR PLATES OF THE ECT

Plate	Total no.	Positive ^a	HVE result		Conf. (%) ^b
			Aspecific	Negative	
<i>Bacillus subtilis</i> , pH 6	24	12	5	7	50
<i>B. subtilis</i> , pH 8	12	10	1	1	83
<i>Micrococcus luteus</i>	21	9	2	10	43
<i>B. subtilis</i> , TMP	24	12	6	6	50
ECT complete	40	15	8	17	37.5

^a Penicillin 7/streptomycin 14.

^b Percentage confirmed.

Table 41.6 CUMULATIVE CONFIRMED^a POSITIVE RESULTS OBTAINED BY THE DIFFERENT PLATES OF THE ECT

Plate	Muscle tissue		Kidney
1. <i>Bacillus subtilis</i> , pH 6	12		55
2. <i>B. subtilis</i> , pH 8	10		62
1 + 2		13	77
3. <i>Micrococcus luteus</i> , pH 8	9		42
1 + 2 + 3		13	78
4. <i>B. subtilis</i> , TMP	12		54
1 + 2 + 3 + 4		15	82

^a Results confirmed by HVE.

plates. With kidneys, the second plate (*B. subtilis*, pH 8) produced the majority of confirmed positive results (62 out of 82). The addition of the first plate raised the number to 77 or the fourth plate (TMP plate) to 75. The third plate (*M. luteus*) raised the number with one extra positive result, and the use of all plates completed the number of 82.

Complaints about the great work-load caused by the use of four plates were received from all participants. In addition, it was reported that reproduction of the TMP plate presented difficulties. With some medium batches the inhibition zone obtained with the sulphonamide standard disc remained smaller than was required according to the prescription (Bogaerts and Wolf, 1980). Sometimes there was no growth at all and new batches had to be prepared.

Discussion

The risks to the consumer from the ingestion of food containing residues of antibacterial drugs have been discussed by many authorities in many countries. Of most antibiotics used in veterinary medicine, the Public Health Council in the Netherlands (1980) has proposed maximum permissible levels in food, in order to ensure that residues are in no way harmful to the health of the consumer. Some examples are presented in *Table 41.1*, in comparison with the detection levels of the ECT. The tolerances are based on known toxicological, allergenic and microbiological effects and the lowest of the three tolerances is proposed. Toxicological as well as allergenic effects will not only be caused by microbiological active substances of drugs, but in most instances also by microbiologically inactive metabolites. If these metabolite concentrations exceed those of the parent drugs, the use of physicochemical methods may be indicated instead of microbiological methods that fail to detect the metabolites. Only for those antibiotics for which tolerances are established on microbiological effects do the microbiological methods seem relevant.

The ECT was advocated as a routine method for testing of frozen muscle samples. Kidneys and livers can also be examined, but interfering factors, such as aspecific inhibitory substances, should be taken into account, especially if samples are deep frozen. The present study shows that even the results of the ECT on fresh kidneys could be confirmed by HVE in only about one-half of the positive results and those on deep-frozen muscle tissue in about

37%. Low confirmation rates (6%) were also obtained by others (Smither *et al.*, 1980), using HVE and thin-layer chromatography combined with bioautography. The reasons for the discrepancies between the results of the ECT and HVE could be a lack of sensitivity of the HVE, a decrease in residue level by deterioration during storage of samples or false positive reactions of the ECT. The first explanation seems less likely because, except for the fourth plate, the same microorganisms are used in both tests. Experiments with spiked samples show comparable sensitivities. The second explanation cannot be excluded, but one of the less stable antibiotics (penicillin) was found in many instances (*Tables 41.4 and 41.5*). Therefore, the third explanation remains to be considered seriously. According to the reports of the 14 participating laboratories, but also according to experiences already published (Schothorst, Leusden and Nouws, 1978), the ECT was laborious and thus expensive. It was calculated that based on examination of 20 samples at once the cost of the ECT per analysis was nearly 3.5 times more than a one-plate method like the Dutch kidney test (Nouws, Schothorst and Ziv, 1979). Consideration of the confirmed positive results obtained with ECT in this study makes one conclude that much effort must be put into application of four plates to raise the number of 62 positive kidneys obtained with one plate (or 77 with two) to 82 with four. It is concluded that the specificity of the ECT and thus its validity is insufficient. Positive results obtained with the ECT need always to be confirmed by another test, e.g. the HVE. As a consequence the test cannot be considered a definitive one, but a screening test only. However, for a screening test the ECT seems too laborious and thus expensive.

Nouws (1981) proposed a very sensitive one-plate test for screening of antibiotic residues. The method used Standard II Nähragar at pH 7.0, containing per ml TMP (0.15 µg) and dextrose (0.004 g) and *B. subtilis* as indicator. Sampling was done with a disc of filter paper impregnated with fluid from the renal pelvis. Results of a preliminary study in 2 Meat Inspection Services comparing the ECT and this test are very hopeful. Out of 20 positive kidney samples, 19 were obtained with the one-plate test and 13 with the ECT. The confirmation result was 80%. The ECT demonstrated inhibitory substances to be present in 5 of the corresponding muscle tissue samples. In conclusion, the development of a simple one-plate screening test is both needed and seems to be possible.

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THE INCIDENCE OF ANTIBIOTIC-RESISTANT *ESCHERICHIA COLI* IN WATER USED ON DAIRY FARMS

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Introduction

A requirement of the Milk and Dairies (General) Regulations (1959) is that water used for dairy purposes must be of a suitable quality and be sufficient for dairy use. As a consequence, Dairy Husbandry Advisory Officers of the Agricultural Development and Advisory Services make field inspections of private farm water supplies every year or 5-7 years, depending on whether the producer retails or wholesales his milk. Samples of the water are submitted routinely to the Microbiology Departments of ADAS and a judgement is made of the suitability of the supply based on the field report and bacteriological results. Any responsibility for the use of these waters for domestic purposes rests with the local Environmental Health Officer (DOE/NWC, 1982).

Water supplies that are well protected and showing only slight faecal contamination are currently allowed to be used without further treatment for dairy purposes. Those that show some evidence of contamination with *Escherichia coli* can be approved, provided the water is boiled or treated with an approved disinfectant. Occasionally, other approved treatment methods are allowed. Supplies that cannot be protected or show gross pollution are not acceptable for dairy purposes. The quality of water used for stock drinking is not controlled and stock frequently have access to polluted supplies, but there was little information on the extent to which different sources were used.

Smith (1970) studied the incidence of *E. coli* containing R-factors in river water and found large numbers of resistant organisms in many British rivers, particularly those receiving insufficiently treated sewage. There was a lower incidence in rivers flowing through predominantly rural areas and none was found in highland regions. Carrington (1979) studied the incidence of antibiotic-resistant coliform bacteria in sewage and concluded that resistance was not acquired during the sewage treatment process and that treatment substantially reduced numbers of coliform bacteria. Grabow, Prozesky and Smith (1974) called for a review of water quality standards because of the incidence of drug-resistant coliforms. Niemi, Sibakov and Niemela (1983) stated that the incidence of resistant strains of faecal coliforms varied among the water samples, without obvious connection with the water source or level

of pollution. Linton (1977) stated that it was not clear whether animal strains of *E. coli* comprised a significant reservoir for man compared with cross-infections within the community, but drew attention to potential cross-infection routes from animals to man and also within animal communities (Linton, 1976). Carrington (1979) postulated that antibiotic-resistant organisms will have no advantage over non-resistant organisms in the aquatic environment in the absence of the relevant antibiotic, but this was contradicted by Levy (1982) who suggested that the resistance determinant, quite apart from its action on antibiotics, may enable the organisms to survive better in the environment. Linton (1981) has said that the indigenous gut flora of animals constituted a highly versatile pool of R-plasmids which under certain conditions could be transmitted to pathogens. The occurrence of numerous cross-reactions among the Enterobacteriaceae including *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella* and others is well known (Jones and Sneath, 1970). A survey of antibiotic-resistant *E. coli* isolated from farm animals in the UK (Jackson, 1981) showed that there was no evidence of any overall increase in incidence during the period 1971-77.

Staff of the Agricultural Development and Advisory Service are in a unique position to study a topic under a variety of geographical conditions in England and Wales, and the requirements of the Milk and Dairies Regulations gave the Microbiology Department an opportunity to examine the incidence of antibiotic resistance among *E. coli* isolated from water supplies in predominantly rural areas. The waters in question were those used for dairy purposes, but it is probable that many of the observations can be extrapolated to other supplies used by rural communities or other classes of stock.

This chapter indicates the types of water supply routinely encountered, the incidence of antibiotic-resistant organisms and the frequency of antibiotic resistance transfer among *E. coli* isolates. Hopefully, the results will aid discussions on the significance of antibiotic-resistant organisms in natural environments and clarify the importance of some cross-infection routes between animals and man.

Methods

COLLECTION OF SAMPLES

Private farm water supplies

Water samples (500 ml) submitted to the microbiology laboratories for the purposes of the Milk and Dairies (General) Regulations were also examined for the presence of antibiotic-resistant *E. coli* and data concerning the nature of the supplies were abstracted from the field reports.

Stock-drinking water

During the course of their routine visits to dairy farms under the Milk and Dairies Regulations, Dairy Husbandry Advisory Officers enquired about the nature of water used for stock drinking by cattle and other livestock on the farm. From the replies received, microbiologists selected a number of farms worthy of further investigation and these were visited, the water supplies inspected and samples were taken for further study.

farm. From the replies received, microbiologists selected a number of farms worthy of further investigation and these were visited, the water supplies inspected and samples were taken for further study.

ISOLATION OF *Escherichia coli*

Water samples were transported in insulated containers and usually tested within 6 h, but always tested within 1 working day. Initial selection and enumeration of *E. coli* in water supplies was by the Most Probable Number technique (DHSS *et al.*, 1969) using formate lactose glutamate medium at 37°C and sub-culturing tubes showing acid and gas into tubes of lactose ricinoleate broth at 44°C. One of the least dilution 44°C positive tubes was selected at random, and a loopful streaked onto eosin methylene blue agar (EMB) or MacConkey agar. The plates were incubated for 24–48 h at 37°C and single typical *E. coli* colonies were randomly selected. The number of colonies selected depended on the initial MPN of *E. coli* per 100 ml as shown below:

MPN	1-14	15-24	25-34	35-49	50-64	65-79	80-94	95-150	151-550	551-1800+
No. of colonies selected	1	2	3	4	5	6	7	8	9	10

The selected cultures were purified by sub-culturing onto nutrient agar (37°C for 24–48 h) or EMB agar or MacConkey agar as appropriate.

IDENTIFICATION OF ANTIBIOTIC RESISTANCE PATTERNS

When pure, a single colony was selected and suspended in sterile quarter-strength Ringer's solution. Confirmation of *E. coli* was checked at this stage by means of the indole test and gas production in lactose ricinoleate broth at 44°C. The suspension was further diluted and sufficient added to pre-dried plates of Diagnostic Sensitivity Agar (Oxoid CM261) that would ensure a

Table 42.1 ANTIBIOTIC CHALLENGE USING OXOID MULTODISKS

<i>Disc 3866E</i>		<i>Disc 6780E</i>	
Antibiotic (code)	Quantity (µg)	Antibiotic (code)	Quantity (µg)
Chloramphenicol (C)	10	Gentamicin (CN)	10
Streptomycin (S)	10	Paramomycin (PM)	10
Tetracycline (TE)	10	Cephaloridine (CR)	25
Neomycin (N)	10	Kanamycin (K)	5
Ampicillin (PN)	10	Nitrofurantoin (F)	50
Furazolidone (FR)	15	Colistin sulphate (CT)	10
Compound sulphonamides (SF)	50	Nalidixic acid (NA)	30
Sulphamethoxazole/trimethoprim (SXT)	25	Spectinomycin (SH)	25

lawn of bacterial growth where colonies were just touching. The plates were dried and then Oxoid Multodisks 3866E and 6780E were added. These contained the antibiotics shown in *Table 42.1*.

In the early part of the survey of private farm water supplies, specially prepared Mastring discs were used. The differences between Oxoid Multodisk and Mastring discs are shown in *Table 42.2*.

Table 42.2 ANTIBIOTICS ON MASTRING DISCS COMPARED TO OXOID MULTODISKS

<i>Antibiotic</i>	<i>Mastring</i>	<i>Oxoid</i>
Furazolidone	10 and 50 µg	15 µg
Ampicillin	25 µg	10 µg
Polymyxin B	300 units	—
Compound sulphonamides	—	50 µg
Sulphamethoxole/trimethoprim	—	25 µg

A control test using the sensitive culture *E. coli* NCTC10418 was set up with each set of plates. The plates were incubated at 37°C for 16–18 h and the antibiotic resistance pattern recorded. Positive cultures were stored on nutrient agar slopes or freeze dried. All resistant isolates were sent to the Bristol laboratory. Resistance to certain antibiotics was not confirmed in a substantial number of isolates when re-checked. This could have been due to repeated sub-culture and/or the effect of freeze drying or incorrect initial diagnosis. All isolates were tested against higher concentrations of antibiotics using Oxoid UI discs or an equivalent concentration of antibiotic.

TRANSFER OF RESISTANCE

Cultures of a nalidixic acid-resistant recipient strain (University of Bristol Medical School) and suspect donors were grown in separate tubes of nutrient broth for 5 h at 37°C. After incubation, 0.8 ml of recipient and 0.2 ml of donor strains were mixed with 9 ml of nutrient broth and incubated at 37°C overnight. A control donor was tested with each batch of isolates.

After incubation, a loopful of broth was streaked onto Diagnostic Sensitivity Agar plates containing nalidixic acid and the antibiotic under test at a concentration of 25 µg/ml. Separate plates were prepared for multi-resistant isolates. Plates were incubated at 37°C for 16 h and examined for growth.

Results

PRIVATE FARM WATER SUPPLIES

Data are currently available from the analysis of survey forms completed by Dairy Husbandry Advisory Officers since 1976. This report shows detail of the period 1976–78 and the antibiotic resistance study work was carried out from January 1977 to February 1979. There are currently approximately 9500 private farm water supplies registered for use for dairy purposes in England and Wales and approximately one-third of these supplies are regarded as being free from *E. coli*. The majority of private farm water supplies would

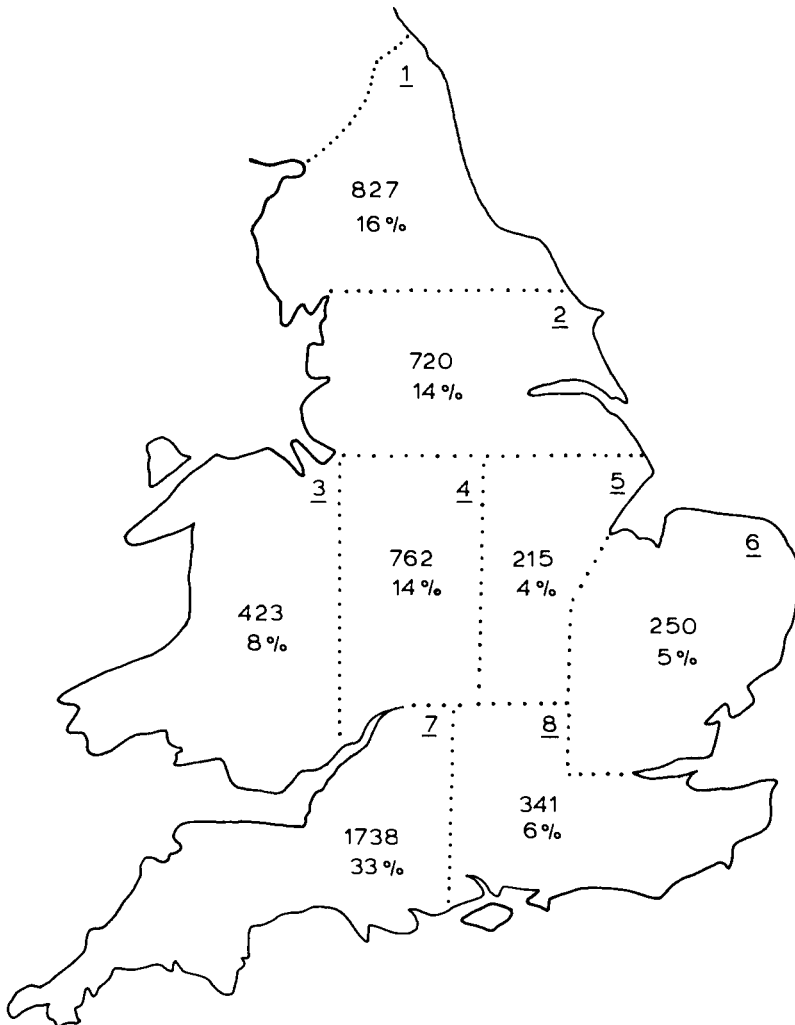


Figure 42.1 Private farm water supplies surveyed by area

also be used for domestic purposes. The distribution of private farm water supplies studied by area is shown in *Figure 42.1*.

In this study, the distribution was chiefly influenced by the location of dairy herds and would not, necessarily, indicate the number of private water supplies used for other purposes in each area. However, this survey probably represents a reasonable cross-section of all types of private water supplies used in the country.

The types of supply are shown in *Table 42.3*, with springs being the predominant type.

It has been the policy of the Ministry of Agriculture, Fisheries and Food over many years to improve private water supplies and those that can be seen to be subject to gross pollution by visual inspection or sampling have largely

Table 42.3 PRIVATE FARM WATER SUPPLY (PFWS)—SOURCES

	Springs	Wells	Source Boreholes	Streams, etc	Others ^a
No. of samples ^b	3161 (60)	764 (15)	1202 (23)	60 (1)	89 (1)

^a Others include rainwater 18, pond 3, etc.^b Figures in parentheses indicate approximate percentage of samples.

been eliminated. In particular, open supplies that cannot be protected against contamination are now rarely used. The remaining adits, streams and ponds were in remote upland areas with low stocking density (mainly sheep). Springs, wells and bores should be protected (MAFF, 1971; 1977), but some were unsatisfactory. *Table 42.4* shows that many wells were shallow and, like

Table 42.4 PFWS—DEPTHS OF WELLS

	Depth (m)		
	Less than 5	5–50	More than 50
No. of supplies ^a	1118 (43)	1076 (41)	417 (16)

^a Figures in parentheses indicate approximate percentage of supplies.

springs, could be prone to surface water contamination and limited natural filtration by the surrounding soil.

In the opinion of the inspecting officers, supplies were frequently near to obvious sources of faecal contamination (*Table 42.5*). These would be largely those near to the homestead, but it should be noted that the geological strata would have a considerable influence on the bacteriological quality of the

Table 42.5 PFWS—DISTANCE FROM SOURCE OF CONTAMINATION

	Distance (m)		
	Less than 10	10–50	More than 50
No. of supplies ^a	1225 (46)	426 (16)	1025 (38)

^a Figures in parentheses indicate approximate percentage of supplies.**Table 42.6** PFWS—GEOLOGY

Formation	No. of supplies ^a
Sandstone	845 (21)
Shale	645 (16)
Limestone	639 (16)
Clay and culm	617 (15)
Chalk	424 (10)
Gravel	239 (6)
Granite	162 (4)
Sand	142 (4)
Marl	105 (3)
Others ^b	221 (5)

^a Figures in parentheses indicate approximate percentage of supplies.^b Others include slate 60, peat 38, siltstone and mudstone 71, loam 52.

Table 42.7 PFWS—LAND USE AROUND SOURCE

	Grassland	Arable	Land use Buildings	Woodland	Others
No. of supplies ^a	2806 (56)	162 (3)	782 (16)	424 (9)	818 (16)

^a Figures in parentheses indicate approximate percentage of supplies.

water. Supplies from fissured rocks such as limestone are frequently found to be contaminated, but the source of contamination can be many miles away. An indication of the geology of the various supplies is shown in *Table 42.6*. It is interesting to note that during the drought of 1976 a large number of supplies were found to deteriorate, probably because of fissuring and increased penetration to the water-bearing strata.

Contamination will be influenced by land use around the source (*Table 42.7*) and land management (*Table 42.8*). The majority of supplies were in grassland, but these were generally in more remote areas and were not subject to intensive grazing and manure spreading.

Table 42.8 PFWS—LAND MANAGEMENT AROUND SOURCE

	Intensive grazing	Management Manure spreading	Neither
No. of supplies ^a	473 (18)	86 (3)	2038 (79)

^a Figures in parentheses indicate approximate percentage of supplies.

ANTIBIOTIC RESISTANCE PATTERNS IN PRIVATE FARM WATER SUPPLIES

In the period January 1977–February 1979 a total of 1576 supplies containing faecal contamination were examined (*Table 42.9*) and 43 (2.7%) were found

Table 42.9 PFWS—SAMPLES TAKEN BY AREA (see *Figure 42.1*)

	Area							
	1	2	3	4	5	6	7	8
No. of samples	93	283	451	152	56	10	465	66

to contain antibiotic-resistant *E. coli*. In general, the most probable number of *E. coli* per 100 ml of water was low and normally less than 250 *E. coli* per 100 ml. From these supplies, a total of 5208 isolates of *E. coli* were checked for their antibiotic resistance patterns and 104 (2%) were found to contain antibiotic-resistant isolates using Oxoid discs. Of these isolates, 58 (56%) also showed antibiotic resistance at the higher antibiotic concentrations using the UI discs.

Resistance to only 5 antibiotics was detected, but multiple resistance was common with 73% of isolates being resistant to 2 or more antibiotics (*Table 42.10*) and 33% to 3 or more. Resistance to the compound sulphonamides occurred with the greatest frequency, followed by streptomycin and tetracycline.

Table 42.10 PFWS—ANTIBIOTIC-RESISTANT *Escherichia coli* ISOLATED

Antibiotic	Antibiotic resistance pattern ^a										No. of times resistance occurred
S	●			●	●	●	●		●	●	38
TE		●			●	●		●	●	●	23
SF			●	●	●		●	●	●	●	51
PN						●	●		●	●	6
C								●		●	5
No. of isolates:											
	1	5	10	23	9	1	1	4	1	2	1

^a ●, Presence of resistance.

Of the 58 isolates, 54 were found to transfer total or partial resistance to the donor strain. Resistance to the compound sulphonamides was transferred with the greatest frequency, while resistance to ampicillin was rarely transferred (*Table 42.11*).

Table 42.11 PFWS—ANTIBIOTIC RESISTANCE TRANSFER AMONG *Escherichia coli* ISOLATES

Antibiotic	No. of times resistance occurred	No. of times resistance transferred	Percentage of times resistance transferred
S	38	13	34
TE	23	11	48
SF	51	48	94
PN	6	1	17
C	5	2	40

STOCK-DRINKING SURVEY

A total of 1261 farms were visited during 1979/80, and details of water usage for stock drinking were recorded. The sources of water used by dairy cattle are shown in *Table 42.12*.

Many farms would use more than one source of water, with predominantly mains and private farm water supplies being used while the cows are housed in the winter period. The size of herd did not appear to greatly influence the source of water used, although the larger herds tended to use more mains

Table 42.12 STOCK-DRINKING WATER (SDW)—NUMBER OF FARMS VISITED

No. of farms	Cow numbers	Source of stock-drinking water					
		Pond	Ditch	River	Mains	Private ^a	Other
583	1-50	40	33	196	379	159	49
484	51-100	44	38	126	393	72	39
146	101-150	4	6	31	126	17	10
20	151-200	1	1	2	16	3	1
10	200+	0	1	1	9	1	0
18	Not known	1	0	13	2	2	1
1261	Total ^b	90 (7)	79 (6)	369 (29)	925 (73)	254 (20)	100 (8)

^a Private = supplies accepted under the Milk and Dairies (General) Regulations (1959).^b Figures in parentheses indicate approximate percentage of source type.

water. The supplies most liable to contamination were often used by other stock; for example, 90 herds had access to ponds and 68% of these were used by other livestock such as horses, beef cattle, sheep and pigs. In the case of rivers and ditches, this was 75% of cases. Samples were taken from 132 farms (11%), as being representative of the national picture, and details are shown in *Table 42.13*.

Table 42.13 SDW—NUMBER OF FARMS SAMPLED

No. of farms	Cow numbers	Source of stock-drinking water					
		Pond	Ditch	River	Mains	Private ^a	Other
41	1-50	6	9	24	25	9	5
60	51-100	14	12	33	39	7	14
20	101-150	2	3	13	13	4	2
1	151-200	0	0	0	0	0	1
0	200+	—	—	—	—	—	—
10	Not known	0	0	7	1	0	1
132	Total ^b	22 (17)	24 (18)	77 (58)	78 (59)	20 (15)	23 (17)

^a See first footnote to *Table 42.12*.

^b Figures in parentheses indicate approximate percentage of source type.

Visiting officers were asked to judge if the water used for stock drinking was subject to pollution by livestock or humans, and in 86% of cases it was judged that one or more of the supplies available to the cattle was subject to pollution and it was these supplies that were sampled. *Table 42.14* shows the sources of pollution, with 6 supplies being subject to farm, domestic and industrial pollution and 29 by farm only.

Table 42.14 SDW—SOURCES OF POLLUTION

No. of supplies	No. subject to pollution ^a	Sources of pollution (where stated)		
		Other farm	Domestic	Industrial
132	113 (86)	60	40	10

^a Figures in parentheses indicate approximate percentage of supplies subject to pollution.

ANTIBIOTIC RESISTANCE PATTERNS IN STOCK-DRINKING WATER

Only 14 supplies (11%) were found to contain antibiotic-resistant *E. coli*. Of these, 12 were from rivers, streams or ditches and 2 from ponds. All were regarded as being subject to obvious pollution; one-half of them by stock on other holdings and one-third by domestic waste. Nine of the supplies were used by stock on other holdings.

All the supplies showed extensive faecal contamination and, in general, contained in excess of 1800 *E. coli* per 100 ml. From these 14 supplies, a total of 127 *E. coli* isolates were selected and tested for antibiotic resistance and 37 (29%) were found to show antibiotic resistance using the Oxoid Multodisks and 28 were confirmed as resistant to the higher concentration of antibiotic

Table 42.15 SDW—ANTIBIOTIC-RESISTANT *Escherichia coli* ISOLATED

Antibiotic	Antibiotic resistance pattern ^a									No. of times resistance occurred
S	●			●		●	●	●	●	17
TE		●			●		●	●	●	22
SF			●	●	●			●	●	16
PN						●	●		●	4
No. of isolates:	1	9	1	3	1	1	1	9	2	

^a ●, Presence of resistance.

on the UI discs (Table 42.15). Resistance to only 4 antibiotics was detected, with resistance to tetracycline occurring with the greatest frequency.

Complete or partial resistance to transfer was shown by 23 of the 28 resistant isolates, with resistance to the compound sulphonamides occurring with the highest frequency (Table 42.16).

Table 42.16 SDW—ANTIBIOTIC RESISTANCE TRANSFER AMONG *Escherichia coli* ISOLATES

Antibiotic	No. of times resistance occurred	No. of times resistance transferred	Percentage of times resistance transferred
S	17	4	24
TE	22	8	36
SF	16	15	94
PN	4	4	100

Discussion

This study concentrated on approximately 9500 private farm water supplies used for dairy purposes, but it has been estimated that up to 2% of the population of Scotland and England, 3% of Wales and 11% of Northern Ireland use private supplies for domestic purposes (DOE/NWC, 1982). This report stated that 78 000 private supplies were known for certain, but estimated that there could be up to 1 000 000 in occasional use and approximately 2 000 000 people regularly drink water from private supplies not controlled by statutory water undertakings. The bacteriological quality of these waters was very variable, but the majority of supplies fell short of modern guidelines. The report stated that proven or suspected cases of water-borne disease were sufficiently frequent to cause concern.

Previously acceptable sources of water used on dairy farms can become polluted, as the stocking density on many farms has increased. Some water supplies which were previously in rough grazing or moorland areas are now located in improved pastures which are subject to intensive grazing. Water supplies used for dairy purposes have been controlled by statute and, in general, are well protected from contamination, but this may not be the case with supplies which are used on other rural premises. The use of *E. coli* as indicators of faecal contamination of recent origin showed that large numbers of private farm water supplies were subject to pollution on occasion. How-

ever, antibiotic-resistant *E. coli* were rarely found in these supplies (less than 3%), and antibiotic resistance was found in only about 2% of *E. coli* isolates. In these few cases, resistance to 5 antibiotics was encountered and multiple resistance was common as was partial or total antibiotic resistance transfer.

A European Community Directive (1980) on the quality of water for human consumption is currently under discussion and will lay down quality standards to be applied to water used for domestic purposes, or food processing, and water affecting the wholesomeness of the foodstuff in its finished form. The biological parameters defined in the directive are the absence of total and faecal coliforms, faecal streptococci and sulphate-reducing Clostridia. It seems likely that this directive will be adopted in the UK and, in the long term, there will be greater control over the use of private water supplies.

There is no statutory control over the water used for stock drinking and it was clear that the majority of dairy cattle had access to polluted water at some time during the year. Transmission of pathogens from water to stock is not uncommon (Smith, Jones and Watson, 1978) and birds can act as vectors of disease organisms to remote areas (Fenlon, 1981). It is unlikely that pigs and poultry in larger units will have access to river and stream water, but our experience is that other private supplies of water are commonly used. The strike of water workers in 1983 forced a number of producers to look for alternative, often unsuitable, sources of water for all types of stock.

From this study it would seem that, although many waters used for stock drinking were polluted, the incidence of antibiotic-resistant organisms was relatively low. When antibiotic-resistant *E. coli* were detected, however, there was a high frequency of antibiotic resistance transfer.

Many river and stream waters could be used for crop irrigation, often in more lowland situations where the pollution would be greater. There is a potential risk to human health from enteric organisms and antibiotic-resistant strains, but this can be minimized as it has been shown that faecal organisms only survive for relatively short periods on leaf surfaces. Advice to growers is to withhold irrigation of crops to be consumed raw for a period of days prior to harvest.

These results indicated that pathways existed whereby antibiotic-resistant organisms could be transferred from man to animals, animals to man and within animal communities, but the number of resistant organisms detected was small. It is probable that these data could be extrapolated to other untreated private water supplies used for domestic purposes and other classes of livestock, but these routes may be of little importance in the acquisition of resistant organisms in rural communities and livestock units.

Acknowledgements

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ANTIMICROBIAL DRUG RESISTANCE IN SALMONELLAE IN BRITAIN—A REAL THREAT TO PUBLIC HEALTH?

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Introduction

In Britain, the most important salmonella serotype in food-poisoning is *Salmonella typhimurium* and infections with this serotype comprise about 27% of all salmonella infections each year (Hepner, 1980). *Salmonella typhimurium* is also the serotype in which antimicrobial drug resistance is most frequently encountered in both isolations from animals (Sojka and Wray, 1980) and humans (Division of Enteric Pathogens, unpublished observations).

In Britain and most European countries, *S. typhimurium* infection in humans is primarily a zoonosis and important food-animal reservoirs are poultry and cattle. For epidemiological studies, the most practical method of differentiating strains of *S. typhimurium* is by phage-typing (Callow, 1959) and long-term studies have shown that, in general, the poultry-associated phage types are drug sensitive, whereas the bovine-associated types are drug resistant. In most resistant strains, resistance is acquired in the animal host before transmission of the pathogens to man and has probably been acquired as a consequence of the use of antimicrobial drugs in the animal species concerned. This is in contrast to the situation in many developing countries in the Middle East, the Indian sub-continent and South America. In these countries, multi-resistant strains of salmonellae have caused extensive outbreaks of severe gastroenteritis, often accompanied by septicaemia (Rowe, 1982; Anon, 1982). In these strains there is no evidence of animal involvement, either in the acquisition of resistance by the strains or in their dissemination. Person-to-person cross-infection appears to be the main method of spread.

This paper describes how drug-resistant strains of 'new' phage types of *S. typhimurium* have appeared and spread in food animals in Britain since 1974. The importance of these events to public health is then assessed.

Multi-resistant *Salmonella typhimurium* in Britain

Prior to 1963, about 3% of *S. typhimurium* were drug resistant, but the incidence of resistance increased dramatically between 1963 and 1969 follow-

ing the appearance and dissemination of a drug-resistant strain of phage type 29 in calves (Anderson, 1968). In 1965, 73% of all isolations of *S. typhimurium* from cattle were caused by this strain but, by 1970, type 29 had disappeared from bovine animals in Britain. The influence of phage type 29 in both cattle and humans resulted in a high incidence of multiply-resistant *S. typhimurium* in 1968 and 1969 (Figure 43.1). However, after the disappearance of type 29 in 1970, for the next six years less than 8% of strains from cattle and 3% of strains from humans were multiply resistant.

Since 1974, resistant strains of 'new' phage types of *S. typhimurium* emerged in cattle, became widely disseminated and caused numerous infections in humans, some of which were fatal. Particularly since 1976, the incidence of multi-resistant strains has increased in both cattle and humans. Because of the influence of drug-sensitive, poultry-associated phage types, this increase

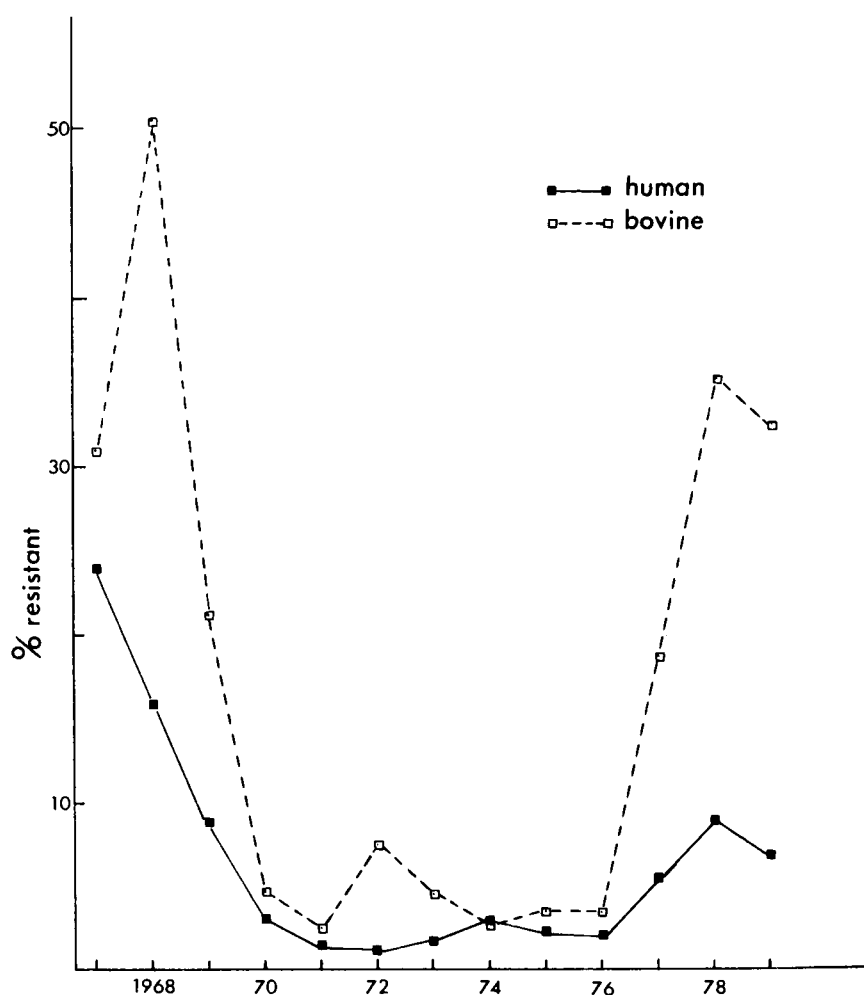


Figure 43.1 Multiple drug resistance in *Salmonella typhimurium* in Britain, 1967-79

is not as pronounced in isolations from humans as in strains from cattle. Nevertheless, the incidence of multi-resistant strains has increased from a baseline of about 1% in 1971/72, to about 10% in 1978 and 9% in 1979, and since 1980 multi-resistant strains have comprised about 8-10% of *S. typhimurium* from humans and about 35% of strains from cattle. The strains have been resistant to many of the therapeutic antibiotics used in human medicine. In particular, some strains have been resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides, tetracyclines and trimethoprim; strains resistant to gentamicin are beginning to emerge.

Of particular importance have been a series of related strains which have appeared and spread in cattle since 1974. These strains belong to phage types 204, 193, 204a and 204c (*Table 43.1*) and since 1977 multi-resistant strains in this series have caused widespread outbreaks of salmonellosis in calf herds.

Table 43.1 APPEARANCE OF *Salmonella typhimurium* PHAGE TYPE 204 AND RELATED TYPES

Phage type	R-type ^a	Date	
204	SuT	1974	January
	CSSuT	1977	June
193	ACKSSuT	1977	December
204a	SuT	1978	October
	CKSSuT	1980	March
204c	CSSuTTm	1979	March
	ACKSSuTTm	1979	October
	SSuTTm	1980	July

^a Resistance symbols: A, ampicillin; C, chloramphenicol; K, neomycin-kanamycin; S, streptomycin; Su, sulphonamides; T, tetracyclines; Tm, trimethoprim.

TYPE 204 AND RELATED TYPES: EPIDEMIOLOGY

Type 204 SuT

In January 1974, a strain of *S. typhimurium* resistant to sulphonamides and tetracyclines (SuT), and with a hitherto unrecognized pattern of reactions with the *S. typhimurium* typing phages, caused a small outbreak among calves in Yorkshire. The phage type was designated type 204. Over the next four years strains of type 204 SuT spread in cattle and caused infections in humans. In particular, strains of this type caused several substantial milk-borne outbreaks in 1975 and 1976. By 1977, type 204 SuT had become the predominant strain in both bovine and human *S. typhimurium* in Britain (Threlfall, Ward and Rowe, 1978a).

Type 204 CSSuT

In June 1977, a strain of type 204 resistant to chloramphenicol, streptomycin, sulphonamides and tetracyclines (CSSuT) caused an outbreak on a farm in Leicestershire. The farm had an extensive calf distribution trade and resulting

from this, type 204 CSSuT became widely disseminated in the following months. Infections reached epizootic proportions and, by October 1977, isolations of type 204 CSSuT were in excess of those of type 204 SuT (Threlfall, Ward and Rowe, 1978a).

Type 193 ACKSSuT

In December 1977, a strain of *S. typhimurium* resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides and tetracyclines (ACKSSuT) appeared in calves in Britain. The strain did not react with the standard typing phages and was assigned to type 193 on the basis of reactions with an ancillary set of phages used for the differentiation of untypable strains.

As with type 204 CSSuT, practices in the calf distribution trade ensured that type 193 ACKSSuT was soon widely disseminated in Britain (Threlfall, Ward and Rowe, 1978b). In 1978 and 1979, strains of type 204 CSSuT and type 193 ACKSSuT had caused outbreaks among cattle in Belgium and Holland. These outbreaks were thought to have resulted from cross-infections following the export of infected calves from Britain to Europe, and were reported to be of unusual severity (Rowe *et al.*, 1979).

Type 204a SuT and CKSSuT

A related phage type, type 204a, was recognized in October 1978. The predominant R-type in this phage type was that of SuT. Subsequently, strains of type 204a SuT spread in cattle and caused numerous outbreaks, particularly in Scotland. By March 1980, type 204a SuT had acquired additional resistance to chloramphenicol, kanamycin and streptomycin (Threlfall, 1982).

Type 204c CSSuTTm and ACKSSuTTm

In March 1979, strains of *S. typhimurium* resistant to chloramphenicol, streptomycin, sulphonamides, tetracyclines and trimethoprim (CSSuTTm) were identified in outbreaks among cattle in Somerset. These strains had atypical reactions with the typing phages, but were derived from type 204, as will be described below. The phage type was designated type 204c.

In the following months, strains of type 204c CSSuTTm caused numerous outbreaks in cattle, and by October 1979 had acquired additional resistance to ampicillin and kanamycin (Threlfall *et al.*, 1980).

TYPE 204 AND RELATED TYPES: DEVELOPMENT

The appearance and dissemination of these related multi-resistant strains was subsequent to the sequential acquisition of resistance plasmids, transposons and carried phages. These processes occurred in the bovine host.

Type 204 SuT

In the progenitor strain of this series, type 204 SuT, Su and T resistances were encoded by independent non-autotransferring plasmids of molecular weight (mol. wt) 4.3×10^6 and 63×10^6 , respectively. The T-resistance plasmid also specified resistance to lysis by many of the *S. typhimurium* typing phages, and when displayed from type 204 SuT, the resultant strain was resistant to Su only and had the phage typing reactions of type 49, a type common in cattle in Britain before 1974.

Type 204 CSSuT and type 193 ACKSSuT

Acquisition of an autotransferring plasmid of compatibility group H₂ which coded for resistance to C, S, Su and T resulted in conversion of type 204 SuT to type 204 CSSuT. The subsequent acquisition of a group I₁ plasmid which coded for resistance to A, K and S, and which also specified resistance to 15 of the typing phages, resulted in conversion of type 204 CSSuT to type 193 ACKSSuT.

Type 204a SuT and CKSSuT

Strains of type 204a SuT were derived from type 204 SuT by the acquisition of a small-plaque temperate bacteriophage which abolished lysis of type 204 by six of the nine typing phages with which type 204 reacted.

Acquisition of a group H₁ R-factor which coded for CKSSuT resulted in the appearance of type 204a CKSSuT.

Type 204c CSSuTTm and ACKSSuTTm

The derivation of type 204c CSSuTTm was a complex process. This involved:

- (1) Loss of the typing phage restriction property from the tetracycline resistance plasmid. This resulted in conversion of type 204 to type 49 without plasmid loss.
- (2) Acquisition of a Tm resistance transposon by the CSSuT group H₂ resistance plasmid.
- (3) Acquisition of a temperate bacteriophage, the presence of which converted type 49 to a new type, subsequently designated 204c.

Six months later, strains of type 204c ACKSSuTTm emerged. These had acquired an additional plasmid of group X, which coded for resistance to A and K.

The stages in the development of these multi-resistant strains are summarized in *Figure 43.2*.

EFFECTS IN CATTLE

Type 204 CSSuT became the predominant strain in cattle in Britain by 1977, but has subsequently disappeared (*Figure 43.3*). Type 193 ACKSSuT peaked

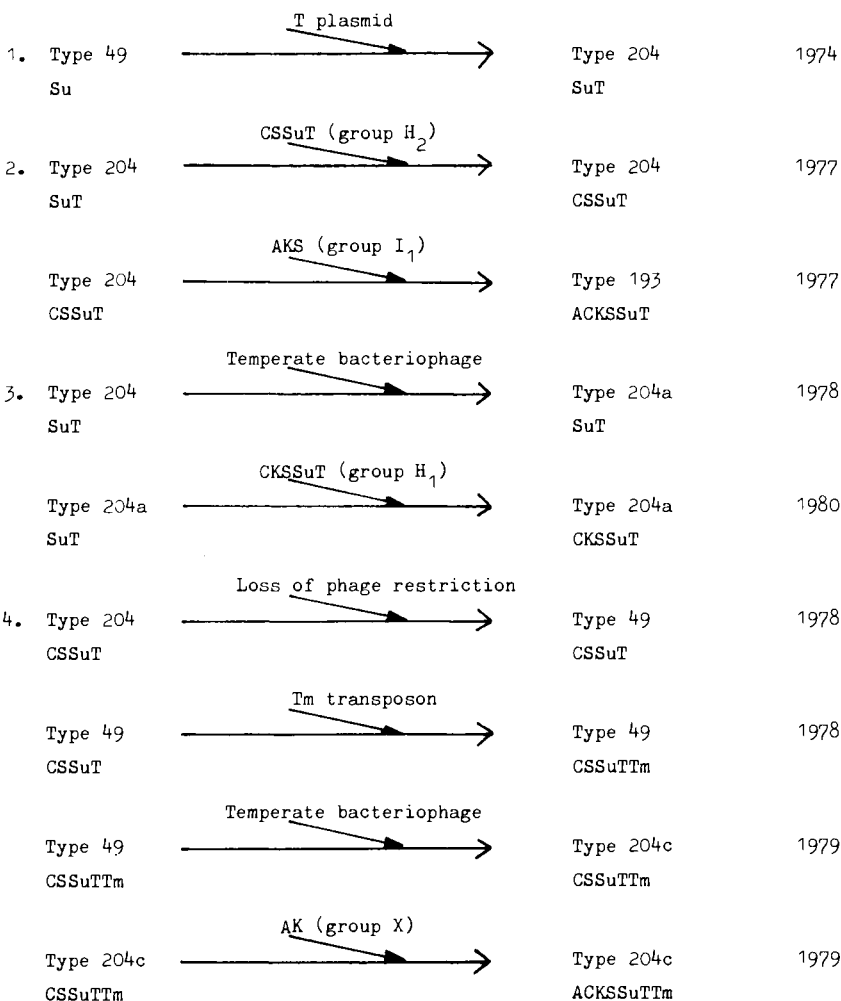


Figure 43.2 Stages in the development of *Salmonella typhimurium* phage types 204, 193, 204a and 204c

in 1978/79, but the frequency of isolation of this strain was greatly reduced in 1980 and had disappeared by 1982. Type 204c, which appeared in 1979, became the predominant multi-resistant strain in cattle during 1980 and remains the most frequently isolated multi-resistant type. In contrast, strains of type 204 SuT have been regularly isolated throughout this period.

As with all strains of *S. typhimurium*, there was a range of symptoms in calves infected with the multi-resistant strains. However, the disease appeared unusually virulent and many reports mentioned severe scouring frequently accompanied by septicaemia. Mortality was high—up to 50% in some calf herds—and economic losses have been considerable. It is possible that the initial rise in prevalence of these multi-resistant strains, followed by their subsequent decline, may be related to changes in selective pressures brought

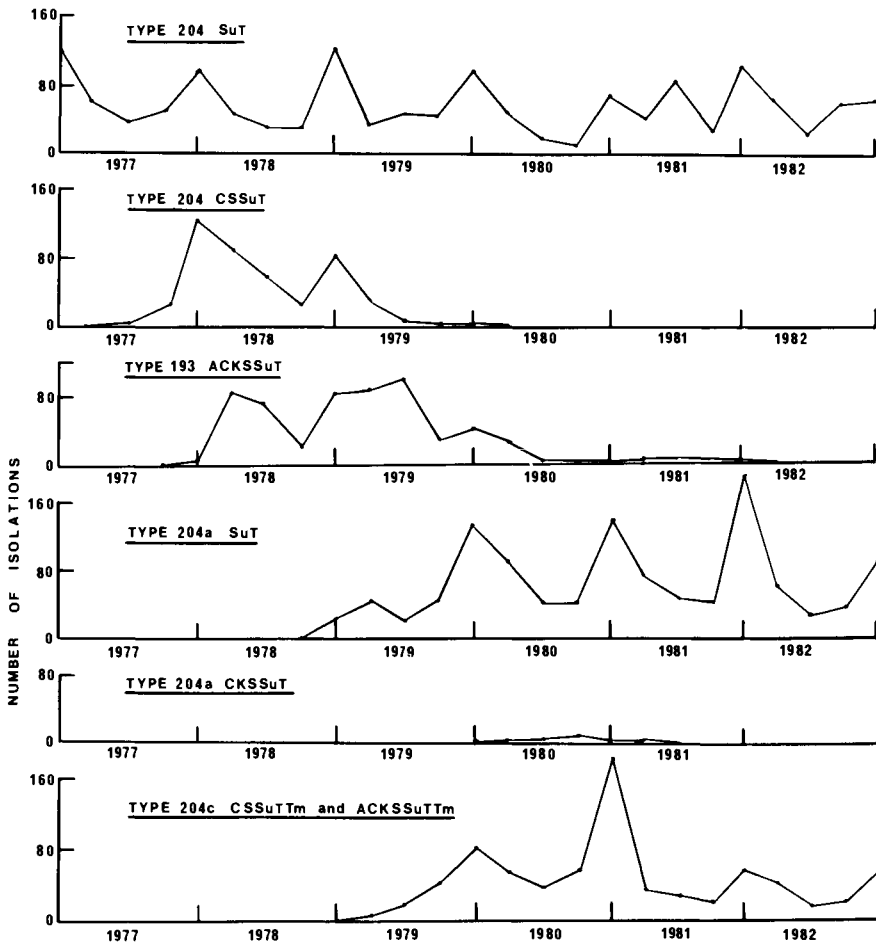


Figure 43.3 *Salmonella typhimurium* types 204, 193, 204a and 204c in cattle, 1977-82

about by the successive use of different antimicrobials in animal husbandry, in attempts to combat the increasing spectrum of resistance in these strains.

EFFECTS IN HUMANS

Among humans, 611 infections with multi-resistant strains of types 204, 193 and 204c have been recognized since 1977 (Table 43.2). In the majority of patients the symptoms were those of mild to moderate enteritis, but several patients suffered from severe diarrhoea which persisted for several weeks. Enteritis was reported as the case of death of 3 patients and there was extra-intestinal spread in at least a further 12 patients. One three-year-old child died with a septicæmic illness following infection with type 193 ACKSSuT, and one patient died of salmonella meningitis following infection with a strain

Table 43.2 HUMAN INFECTIONS WITH MULTI-RESISTANT *Salmonella typhimurium* OF PHAGE TYPES 204, 193, 204a and 204c, 1977-82

Year	Type 204	Type 193	Type 204c			Type 204a	Totals
	CSSuT	ACKSSuT	CSSuTTm or	ACKSSuTTm	SSuTTm	CKSSuT	
1977	37	4	0		0	0	41
1978	51	89	0		0	0	140
1979	15	94	20		0	0	129
1980	0	29	102		16	48	195
1981	0	0	63		2	5	70
1982	0	6	30		0	0	36
Totals	103	222	215		18	53	611

Source: strains referred to Division of Enteric Pathogens.

of type 204c. These patients did not respond to treatment with the limited range of antimicrobial drugs left available to the physician.

Trimethoprim resistance

Since 1979 there has been a substantial increase in isolations of trimethoprim-resistant salmonellae in Britain ((*Figure 43.4*). This increase has been particularly evident in *S. typhimurium* and has resulted mainly from the appearance and spread of Tm-resistant strains of three phage types 18, 170 and 204c (Ward, Rowe and Threlfall, 1982).

TYPES 18, 170 AND 204c

Type 18

Tm-resistant strains of type 18 appeared in cattle in the south-east of England in 1979 and have subsequently caused many human infections. In humans, about 60% of strains of this phage type isolated in 1980 and 1981 were Tm resistant and among Tm-resistant strains, the predominant R-types were those of SuTm and SSuTTm.

Type 170

Tm-resistant strains of type 170 appeared in cattle in Derbyshire in 1981 and in man a few weeks later. In 1981, 54% of type 170 strains from cattle and 64% of strains from humans were Tm resistant. The predominant R-types were those of SuTm and SSuTm.

Type 204c

The appearance and spread of strains of phage type 204c has been described above. All strains of type 204c so far isolated have been Tm resistant and the most common R-types have been those of CSSuTTm, ACKSSuTTm and SSuTTm.

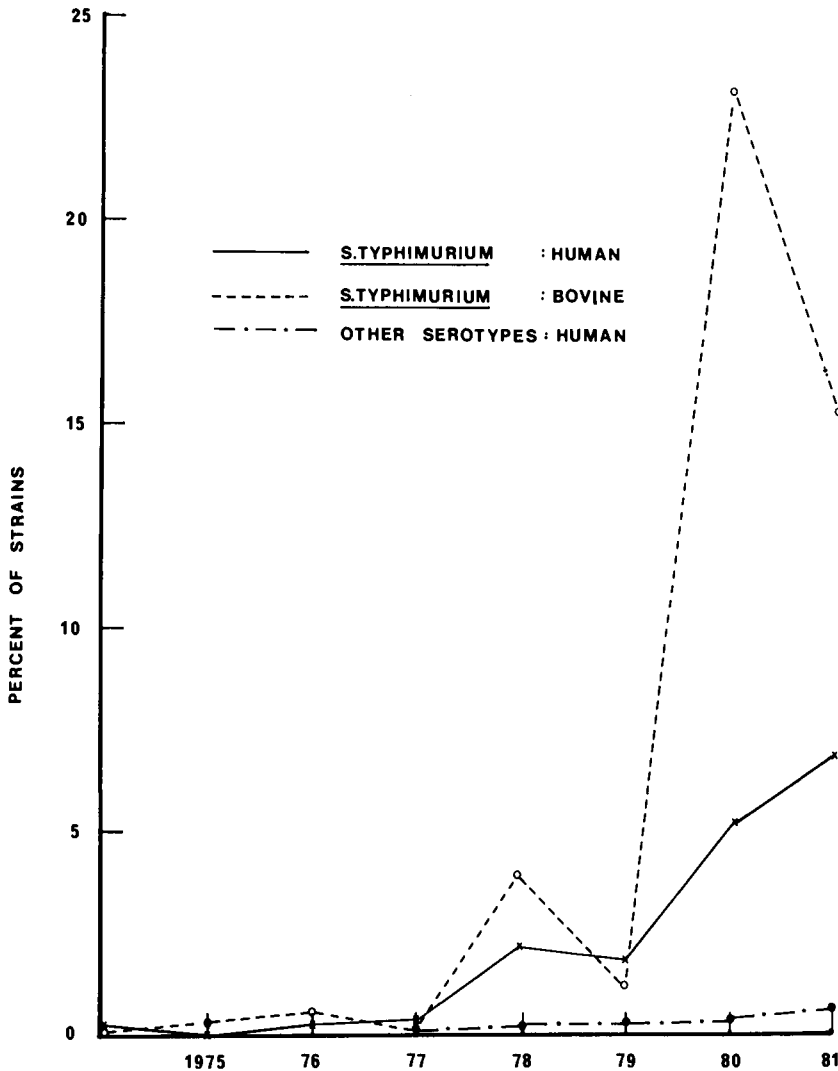


Figure 43.4 Trimethoprim resistance in salmonellae in Britain, 1974-81

TRIMETHOPRIM RESISTANCE PLASMIDS

In both types 18 and 170, trimethoprim resistance was encoded by a non-autotransferring plasmid which also specified resistance to sulphonamides. These plasmids were of compatibility group 3, in accordance with the scheme of Smith (1975) for the classification of non-autotransferring plasmids (Threlfall *et al*, 1983). In all type 204c strains, Tm resistance was coded for by an autotransferring group H₂ plasmid, which also conferred resistance to C, S, Su and T. Thus, all isolations of type 204c were probably derivatives of a single strain which has become widely disseminated in calves. Similarly,

although not all strains of types 18 and 170 were Tm resistant, the plasmid characterization studies have demonstrated that, within each of these phage types, Tm-resistant strains were probably clonal.

EFFECTS IN HUMANS

Since 1979, there have been 832 infections in humans caused by Tm-resistant strains of phage types 18, 170 and 204c (*Table 43.3*). These have comprised about 4% of total human *S. typhimurium* strains referred to the Division of Enteric Pathogens (DEP) since 1979.

The appearance and spread of Tm-resistant strains of types 18, 170 and

Table 43.3 HUMAN INFECTIONS WITH TRIMETHOPRIM-RESISTANT *Salmonella typhimurium* OF PHAGE TYPES 18, 170 AND 204c, 1979-82

Year	Type 18	Type 170	Type 204c	Totals
1979	47	0	20	67
1980	67	0	118	185
1981	67	150	65	282
1982	86	182	30	298
Totals	267	332	233	832

204c followed an intensive promotional campaign to encourage the use of trimethoprim-containing products, after it had been reported that trimethoprim resistance was rare in salmonellae (West and White, 1979). Trimethoprim is one of the drugs of choice for the treatment of typhoid fever and salmonella septicaemia and there is no doubt that its value is being diminished by the proliferation of Tm-resistant strains of *S. typhimurium* which have originated in cattle.

Gentamicin resistance

Gentamicin is used to treat septicaemia and other severe systemic infections such as osteomyelitis, endocarditis and meningitis. Almost all gentamicin-resistant salmonellae isolated from humans in Britain have been confined to persons who have either acquired their infections abroad, or have had contact with persons from countries where gentamicin-resistant strains have caused extensive outbreaks (Frost *et al.*, 1982). In food animals, the first report of gentamicin resistance was in a strain of *S. typhimurium* isolated in 1982 from a 4-week-old pig. The strain was also resistant to A, C, K, S, Su and T and resistances were plasmid-encoded (Sojka *et al.*, 1982). Restraint in the prescribing of antibiotics was urged.

STRAINS FROM CHICKENS

In the DEP, gentamicin resistance has been observed in strains of *S. typhimurium* isolated from chickens in 1981 and 1982.

Two phage types have been identified, types 80 and 160, and the strains have been resistant to gentamicin and streptomycin (GS) with an MIC to gentamicin of 16 µg/ml. Preliminary studies have indicated that these resistances are not specified by plasmids.

STRAINS FROM HUMANS

In 1982, 7 strains of R-type GS and of phage type 160 were isolated from humans. These strains were indistinguishable from those isolated from chickens, and the possibility must be considered that gentamicin-resistant strains of avian origin have entered the food-chain. This is particularly disturbing, since chicken-associated phage types of *S. typhimurium* are becoming increasingly important in food-poisoning in Britain and now constitute a high proportion of strains isolated each year.

Discussion

Antibiotics are not recommended for the routine treatment of salmonella enteritis and there is no evidence to suggest that, in Britain, resistant strains of animal origin have enhanced virulence for humans. Therefore, it is quite justifiable to ask whether, in Britain, antimicrobial drug resistance in salmonellae does pose a real threat to public health.

To answer this question, it must be remembered that, in humans, extra-intestinal infections with strains of animal origin do occur and are not confined to the elderly, or to debilitated patients. Since 1976, the probability of a human *S. typhimurium* infection being caused by a multi-resistant strain has increased by about 8-fold; thus, the possibility of extra-intestinal infections with multi-resistant strains has also increased. In systemic salmonellosis, antimicrobial therapy is essential and in this respect the mass emergence of strains resistant to such valuable drugs as ampicillin, chloramphenicol, kanamycin and trimethoprim is particularly disturbing.

It is considered that, if unchecked, the emergence and spread of multi-resistant strains in food animals can have more serious consequences to human health, particularly if the strains become better adapted to epidemic spread in humans. Reduction in the prophylactic use of antibiotics in food animals would probably reduce the likelihood of the emergence of resistant strains. If this was combined with realistic measures to prevent the dissemination of infected animals, then some reduction in the incidence of drug-resistant strains in humans may be expected.

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ANTIBIOTIC-RESISTANT BACTERIA IN FOOD OF MAN AND ANIMALS

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Introduction

Antibiotics are used for treatment, growth promotion and prophylaxis of humans, animals and cultivated plants. Their widespread usage has led to the emergence of resistant bacteria in all countries of the world. Considerable attention has been given to the types and quantities of resistant bacteria associated with man and animals. Of particular concern are those pathogens, which have recently emerged, which are resistant to previously effective antimicrobial therapy (Levy, 1982). Much less effort has been extended towards examining the levels and kinds of resistant bacteria associated with agriculture and, in particular, the fruits and vegetables that humans and animals consume. While most of the organisms associated with edible products should be non-faecal in origin (at least with good sewage processing, as in the developed world) this is not necessarily so, since it can depend on the fate of sewage sludge and animal excrement. And, in fact, food has been shown to be the source of *Pseudomonas* infections in hospitals (Shooter *et al.*, 1971; Remington and Schimpff, 1981). This is particularly a problem among the immunocompromised patients. In developing countries, human faecal contamination may be more common because of less-developed sanitation processes.

Examination of over 1200 faecal samples from man and animals in the Boston area revealed high levels and high frequency of resistance to one or more antibiotics among ambulatory individuals who were not taking an antibiotic (Marshall *et al.*, 1981). Moreover, the only detectable difference between a faecal sample from an individual taking an antibiotic and one who was not was a larger number of different resistances present in the former. Furthermore, tetracycline resistance was found at 5-fold higher frequency than recorded in a study performed 5 years previously (Levy, FitzGerald and Macone, 1976).

In order to understand the origin of so many resistant bacteria, we examined the level and kinds of resistant organisms being ingested in association with fruits and vegetables (Marshall *et al.*, in preparation). Our studies showed a large number of antibiotic-resistant Gram-negative, bile salt resistant bacteria contaminating vegetables and fruits being sold or offered in fruit

stalls, supermarkets and restaurants in the Boston area. We suggest that such foods may be a source of spread of resistant bacteria in many areas of the world.

Microbiological testing of vegetables and fruits

Nine different vegetables and 5 different fruits were tested. The vegetable samples were aseptically cut and then homogenized in a 10% sterile buffer suspension (weight/volume). The suspension was appropriately diluted and plated onto MacConkey agar plates. Subsequent to growth, these 'master' plates were replica-plated to the same agar containing antibiotics.

Thirteen tomatoes from fruit stalls and supermarkets were examined. The range of Gram-negative bacteria was from 1.7×10^4 /g to 2.1×10^5 /g, with a

Table 44.1 RESISTANT GRAM-NEGATIVE BACTERIA FROM MARKET PRODUCE

<i>Vegetable</i>	<i>No. examined</i>	<i>Mean titre $\pm$ s.d.</i>	<i>Colony type (mean %)</i>	<i>Mean % ≥ 1 resistance</i>	<i>Mean % ≥ 2 resistance</i>
Carrot	3	$1.1 \pm 8 \times 10^6$	lac ⁺ 25.2	53.4	31.4
			lac ⁻ 74.8	87	76.1
Celery	11	$6.2 \pm 7.4 \times 10^5$	lac ⁺ 6.6	84.8	83
			lac ⁻ 93.4	81.5	63
Cucumber	12	$4.8 \pm 4.9 \times 10^5$	lac ⁺ 9.0	39.7	20
			lac ⁻ 91	82.9	80.5
Lettuce (whole)	9	$5.0 \pm 5.9 \times 10^5$	lac ⁺ 0.6	N.A.	N.A.
			lac ⁻ 99.4	73.3	46.7
Lettuce (outside leaves)	3	$4.7 \pm 4 \times 10^4$	lac ⁺ 26.2	64.3	53
			lac ⁻ 73.8	81.4	49.3
Lettuce (inside leaves)	4	$5.6 \pm 5.7 \times 10^1$	lac ⁺ 0	N.A.	N.A.
			lac ⁻ 100	100	100
Pepper	10	$2.68 \pm 4.7 \times 10^6$	lac ⁺ 15.4	63.3	56.4
			lac ⁻ 84.6	80	63.2
Tomato	13	$9.4 \pm 8.6 \times 10^4$	lac ⁺ 11.7	73.9	50.7
			lac ⁻ 88.3	87.9	73.6

mean of about 10^5 /g (*Table 44.1*). The distribution of lactose fermenting (lac⁺) and non-fermenting (lac⁻) bacteria varied with each tomato; on the average about 90% were lac⁻. Of the lac⁺ colonies isolated, 74% were resistant to at least 1 of 8 antibiotics tested: tetracycline (Tc), ampicillin (Ap), kanamycin (Kn), gentamicin (Gn), cephalothin (Kf), streptomycin (Sm), nalidixic acid (Na) and chloramphenicol (Cm). Fifty-one per cent were resistant to 2 or more drugs. Resistance to 7 antibiotics was found in an apparent *Klebsiella* at a titre of 4×10^3 /g, which represented 8% of the total bacteria (*Table 44.2*). Other tomatoes showed 3–33% multiply-resistant lac⁺ strains. Among the lac⁻ isolates, resistances were higher (74–88%) (*Table 44.1*); 9 different antibiotic resistance combinations were found among the lac⁻ col-

Table 44.2 MEAN TITRE OF SELECTED PREDOMINANT MULTIPLE RESISTANCE

Vegetable	Colony type	$\geq 2^R$	$\geq 3^R$	Patterns $\geq 4^R$	(cfu/g) $\geq 5^R$	$\geq 6^R$	$\geq 7^R$
Carrot	lac ⁺	6.7×10^4	—	—	—	—	—
	lac ⁻	—	1.1×10^5	6×10^4	4×10^4	—	—
Celery	lac ⁺	5.4×10^3	2.1×10^4	3×10^4	—	—	—
	lac ⁻	—	—	1.4×10^4	4×10^3	—	7.1×10^4
Cucumber	lac ⁺	1.5×10^4	—	1.0×10^3	—	—	—
	lac ⁻	5.3×10^3	7.3×10^4	1.8×10^5	2.1×10^5	—	—
Lettuce (whole)	lac ⁺	—	1.7×10^3	—	—	—	—
	lac ⁻	5.7×10^3	1.6×10^5	3.5×10^5	—	—	—
Pepper	lac ⁺	2×10^4	8×10^3	1×10^3	1.6×10^4	—	—
	lac ⁻	3.9×10^4	2×10^4	2.5×10^6	7.3×10^3	2.8×10^4	—
Tomato	lac ⁺	1×10^3	1.5×10^2	—	1×10^2	1×10^3	4×10^3
	lac ⁻	2.8×10^4	5.9×10^4	4.5×10^4	7.0×10^4	4×10^4	—

onies at frequencies from 1.1% to 99% of all organisms isolated from a single tomato. Among the higher percentages were resistance to 5 and 6 antibiotics, in particular to Ap, Kf, Tc, Sm, Na and Cm. Among the genera of bacteria isolated were *Enterobacter*, *Klebsiella*, *Serratia* and *Pseudomonas*.

Sixteen lettuces were examined, 7 of which were separated into inside and outside leaves and 9 were tested as whole lettuce samples. The inside of the lettuce contained 1000-fold fewer cells/g than did the outside: 10^1 (inside) v. 4.7×10^4 (outside). Moreover, the lac⁺ strains were almost exclusively on the outside leaves. They were found at 76% on one lettuce and 2–4% on others. More than 90% of all organisms were lac⁻, of which 73% were resistant to 1 or more antibiotics. Resistance to more than one drug was found at a mean of 47% for all organisms cultured. Among the lac⁻ colonies in whole lettuce samples, Ap/Kf resistance was common, together with resistances to Cm, Na and Sm. Resistance to Ap, Kf, Gn and Kn was also found among the lac⁺ colonies. The kinds of organisms included *Enterobacter agglomerans*, *E. cloacae*, *Klebsiella pneumoniae*, *Serratia liquefaciens* and *Escherichia coli*.

The range of bacteria isolated from 12 cucumbers was 1.2×10^3 to 1×10^6 with a mean titre of $4.8 \pm 4.9 \times 10^5$. Ninety-one per cent were lac⁻; lac⁺ bacteria ranged from 3% to 10%. Eighty per cent of the lac⁻ group were resistant to multiple antibiotics. A mean titre of 2×10^5 organisms was found resistant to 5 drugs (Table 44.2). Resistance to 4 antibiotics (Ap, Kf, Cm, Na) was found at relatively high titre (10^3) among the lac⁺ bacteria on 1 cucumber.

Ten peppers weighing from 86 to 236 g were analysed. Fifteen per cent of the bacteria were lac⁺ at titres of 10^3 – 2×10^5 /g; 56% of these were resistant to more than one antibiotic. The majority of resistant organisms were lac⁻, with resistance ranging from 1.5% to 100%; a mean of 60% was multi-resistant. Resistance to all 8 antibiotics was found in the lac⁻ group at titres of 10^3 /g. On 1 pepper, all lac⁻ organisms were resistant to 6 drugs. Of the lac⁺ organisms, 3–5 antibiotic resistances (Ap, Kf, Kn, Sm, Gn) were found in from 4% to as high as 100% of the total organisms.

Analysis of 11 celery samples showed a mean titre of bacteria at 6×10^5 /g,

with a range from 3×10^4 to 2×10^6 . Most of the organisms (93%) were lac^- , in contrast to relatively less amounts of lac^- (75–85%) associated with tomatoes and peppers. Still, in any one sample, up to 22% of the bacteria were found to be lac^+ . The frequency of multi-resistance among the lac^+ ranged from 35% to 100% and was higher than that for lac^- : 83% as compared with 63%. Resistance to any antibiotic at $> 10\%$ frequency was found at 55–100% of all the colonies examined in any preparation. As was seen with the other vegetables, large numbers of resistances were found in the lac^- , including those to the aminoglycosides, the β -lactams, Cm and Tc. One celery contained 13.2% lac^- colonies resistant to all 8 antibiotics tested. The largest number of resistance phenotypes encountered among the lac^+ were found on celery samples. Ten different combinations to all drugs except nalidixic acid were found among lac^+ strains. Titres ranged from 1×10^2 to 5×10^4 , the latter to Ap, Tc, Gn and Kn.

From 3 carrots tested, the mean bacteria titre was $1.1 \pm 8 \times 10^6/\text{g}$ with about 25% lac^+ . Resistance to 1 or more antibiotics ranged from 43% to 86% (mean 70% for all organisms present). Multiple resistance was particularly high among the lac^- strains (almost 80%), and 31% of lac^+ isolates were multi-resistant. The most common resistance phenotype among the lac^+ colonies was to Ap and Kf, presumably *Enterobacter*, at a level of about $1 \times 10^5/\text{g}$. Multiple resistance characterized the lac^- isolates with organisms resistant to Ap, Kf, Cm and Kn at 5–10%.

Examination of a single parsnip and several radishes also showed 17% and 33% of the lac^+ and 33% and 64%, respectively, of lac^- colonies resistant to 2 or more drugs.

Examination of several onions demonstrated no bacterial growth, a result presumably due to the inactivation of bacteria by the onion juices.

Grapes which were bought at a stall also showed no growth. Four tangerines were swabbed and the titre was between 6×10^3 and 1×10^4 ; 85–95% were lac^- type. Thirty-eight per cent of these organisms were resistant to 1 drug and 15% were multi-resistant (2–50% of lac^- organisms). Oranges were swabbed and showed titres of 10^2 – 10^3 per fruit, with about 40% resistant to more than one antibiotic. On 3 oranges, 90% of bacteria were multi-resistant *Pseudomonas aeruginosa*. The bananas had higher titres (1×10^4) with primarily single resistance. A single apple tested gave no growth.

The finding of multiply-resistant organisms in the food suggests contact of these agricultural products with antibiotics. The range of bacterial contaminants was 10^5 – $10^6/\text{g}$ with 70–90% resistance. Although the lactose-fermenting varieties were fewer in number, they were still at detectable and significant levels between $10^2/\text{g}$ and $10^4/\text{g}$. Most of these also carried multiple resistance. Since most individuals eat from 20 to 100 g of any one vegetable, the total amount of organisms ingested is substantial. It was estimated that in an average salad of tomato, lettuce and cucumber, about 10^9 total bacteria would be ingested.

In preliminary studies, transfer of resistances from these organisms to a laboratory strain of *E. coli* was tested. Twenty-two lac^- strains were tested, looking for all possible transfers (about 115 possibilities). Fifteen gave evidence of transferring Kf, but only 1 appeared stable; others were lost on sub-culture. One *P. aeruginosa* transferred Km stably. Six lac^+ strains were examined, looking for a total of 9 possible transfers. Two transferred Kf, but

the transconjugants grew poorly on sub-culture. One transferred Sm, but this was also unstable. Examination of total cellular DNA in agarose gels easily showed multiple plasmids in all organisms so tested. In general, large plasmids, which could be transferable, were seen. Additional transfer studies and speciation are in progress.

Effect of human refuse on gastrointestinal flora of wild primates

This analysis of food suggested that propagation of antibiotic-resistant bacteria and their plasmid pools in man and animals could be occurring by the daily ingestion of these resistant organisms in their food. Since antibiotic-resistant bacteria were so commonly found among ambulatory humans, it was not possible to examine the effects of food on human faecal flora. However, an experimental situation was found where this type of examination was possible.

In collaboration with Glenn Hausfater of the Division of Biological Sciences at Cornell University, we studied enteric bacteria in wild baboons in Kenya. Three groups of baboons living in Amboselli Park in Kenya were examined. Two groups, Alto's and Hook's, lived in areas away from humans,

Table 44.3 ANTIBIOTIC RESISTANCE AMONG WILD BABOONS IN AMBOSELLI PARK, KENYA

Baboon group	No. faecal samples	Percentage completely sensitive	Percentage with multiple resistances	Percentage with $\geq 20\%$ of resistant colonies/sample
Alto's	40	53	5	23
Hook's	25	64	8	8
Lodge	18	6	88	71

except for some local tribesmen who occasionally grazed their cattle. The third group of baboons from the same species lived in proximity to the tourist lodge. These baboons, in addition to roaming areas of the wild, would raid refuse pits near these lodges. Faecal samples were collected from baboons from each of these three groups and were examined for the types of organisms and for the kinds and levels of resistance. These studies demonstrated a greater variety of lactose-fermenting and non-fermenting bacteria among the group feeding on human refuse. Moreover, a greater frequency of multiply-resistant organisms was found (*Table 44.3*) (Rolland *et al.*, submitted). Eighty-eight per cent of the faecal samples from the Lodge group contained multiply-resistant coliforms, as compared to 5–8% in the other two groups. Although the levels were generally below 10% for any one kind of resistance, the total number of resistant bacteria was above 10%. In 71% of the Lodge samples, > 20% of all bacteria were resistant. There was no consistent pattern, but 3 and 4 resistances were not uncommon; moreover, these resistances could be transferred to laboratory strains of *E. coli*.

This unusual study demonstrated that faecal flora of primates can be altered by feeding habits. Furthermore, the results suggest that feeding on human waste products caused an alteration in the faecal flora to the selection of multiply-resistant organisms. They point to human refuse as a source of

antibiotic-resistant organisms and as a source of cross-colonization among animals and man.

It is difficult to determine the origin of the resistant bacteria in food, but one source could be the use as fertilizers of excrement of animals being fed these drugs. Another source could be contamination during processing. In some areas, it might be argued that sludge from human sewage has been used as land fill and may be a source. A study in Holland suggested that *Salmonella* could be followed from slaughtered animal to human consumer, to the environment, back to animal and finally back to human consumer through cross-contamination in all areas of the environment (Edel, van Schothorst and Kampelmacher, 1976).

Our study suggests that the same selection by antibiotics which has been documented in man and animals is occurring among cultivated plants. They also allow another interpretation of data previously published comparing vegetarians and omnivores. The expectation was that meat eaters might have higher levels of resistant coliforms. This was not the finding. In fact, faecal samples from vegetarians showed higher frequency of resistant bacteria (Guinee, Ugnèto and van Leeuwen, 1970). Moreover, it has been documented that contamination by animal bacteria usually occurs during the preparation of the meat, not in eating it (Linton *et al.*, 1977). If vegetables and fruits bear resistant bacteria and they appear to have a selective advantage over sensitive ones, the opportunity to ingest large amounts of resistant bacteria could be greater among the vegetarian group. In a broader sense, the spread of resistant strains through food products normally not cooked offers a means of continued re-infection of humans and animals with resistant bacteria already selected in the environment.

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THE ECOLOGY OF ANTIBIOTIC-RESISTANT BACTERIA IN ANIMALS AND THEIR ENVIRONMENT

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The majority of studies on antibiotic-resistant bacteria in animals have concentrated on certain groups of pathogenic organisms and a few members of the indigenous flora of the animals' gut. The former include *Salmonella* spp., *Staphylococcus aureus*, *Pasteurella multocida* and *Pasteurella haemolytica*. Most work on the indigenous flora of the gut has been concerned with *Escherichia coli*. In considering the ecology of drug resistance, however, it is necessary to review the role of all species of bacteria carrying R-plasmids and the range is extensive (*Table 45.1*). Many of the bacteria listed in the table have been found in association with man and his environment, but comparatively little information is available from animals. In this chapter, studies on the ecology of antibiotic-resistant bacteria in animals and their environment are summarized.

Table 45.1 NATURAL BACTERIAL ISOLATES THAT CONTAIN TETRACYCLINE RESISTANCE PLASMIDS

<i>Achromobacter liquefaciens</i>	<i>Pseudomonas aeruginosa</i>
<i>Bacillus cereus</i>	<i>Salmonella panama</i>
<i>B. stearothermophilus</i>	<i>S. paratyphi B</i>
<i>Bacteroides fragilis</i>	<i>S. typhi</i>
<i>Campylobacter fetus</i> subsp. <i>jejuni</i>	<i>S. typhimurium</i>
<i>Enterobacter cloacae</i>	<i>Shigella dysenteriae</i>
<i>Escherichia coli</i>	<i>Sh. flexneri</i>
<i>Haemophilus influenzae</i>	<i>Sh. sonnei</i>
<i>Klebsiella pneumoniae</i>	<i>Staphylococcus aureus</i>
<i>Proteus mirabilis</i>	<i>Streptococcus agalactiae</i>
<i>P.morganii</i>	<i>Strep. faecalis</i>
<i>P. rettgeri</i>	<i>Strep. faecalis</i> sub-sp. <i>zymogenes</i>
<i>Providencia</i> sp.	<i>Vibrio cholerae</i>

Source: from Chopra, Ball and Shales (1983), by courtesy of Academic Press.

Antibiotic resistance in *Salmonella typhimurium* and *Escherichia coli*, in calves fed milk substitute and their environment

Apart from an outbreak in the 1960s, multi-resistant strains of *Salmonella typhimurium* were rarely encountered in animals in the UK before 1976 (Sojka

and Wray, 1980; Linton, 1981; 1982). Since then the incidence rose considerably due principally to an epidemic in calves of a series of closely related phage types. The epidemic proved extremely serious because the various clones of resistant phage types which arose were widely disseminated throughout England, Wales and Scotland, chiefly due to the movement of calves. Infection occurred not only in calves, but to a lesser extent in adult cattle and other farm animals; infections were also found in man.

Epidemiological studies of infection by chloramphenicol-resistant phage types of *S. typhimurium*, in 16 batches of calves (a total of 495 calves) brought into a veal unit in Somerset, were undertaken in 1979 and 1982 (Hinton *et al.*, 1983). Each batch was monitored over a 4- or 5-week period, from the time

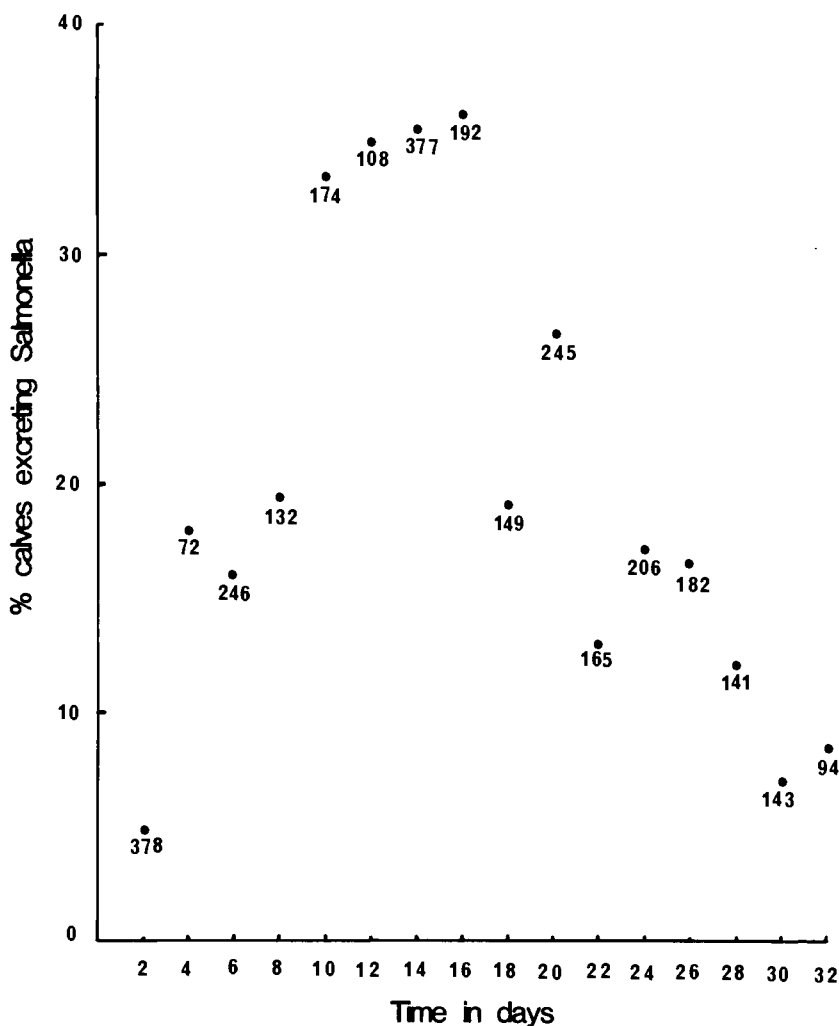


Figure 45.1 The incidence of *Salmonella typhimurium* excretion in 12 batches of calves. The numbers at each point indicate the number of calves sampled on the corresponding day (from Hinton *et al.*, 1983)

they arrived at the rearing farm up to the time they left for export. Two phage types of *S. typhimurium* were involved—DT 193, in 1979, with resistance pattern Cm, Sm, Su, Tc, Ap, Km (see footnotes to *Tables 45.3* and *45.4* for symbol interpretation) and DT 204c, in 1982, with a resistance pattern similar to DT 193 but with the addition of trimethoprim resistance. The incidence of excretion in each batch of calves followed a regular pattern and the data for 12 batches, studied in 1982, are presented (*Figure 45.1*). On intake, the incidence was either zero or relatively low; it then rose to a peak in the second or third weeks, before declining to low levels by the end of the fourth week (Linton, Timoney and Hinton, 1981; Hinton *et al.*, 1983).

Escherichia coli, isolated from rectal swabs taken from calves in the first batch in 1979, were also studied (Linton, Timoney and Hinton, 1981). In contrast to the epidemiology of the *S. typhimurium*, the proportion of antibiotic-resistant *E. coli* carrying patterns of resistance similar to those exhibited by *S. typhimurium* phage type 193 increased with time and persisted in the dominant faecal flora after the salmonellae had virtually disappeared. On intake, 15% of all isolates of *E. coli* on unselective media were chloramphenicol resistant (*Table 45.2*); these were isolated from only 2 of the 40

Table 45.2 THE PERCENTAGES OF *Escherichia coli* RESISTANCE TO EACH OF 6 ANTIBACTERIAL AGENTS ON 11 SAMPLING OCCASIONS FROM CALVES REARED INTENSIVELY FOR VEAL

Antibacterial agent	Days of sampling											Percentage of all isolates
	1	2	6	9	13	15	21	23	27	30	34	
Ampicillin	38.5 ^a	24	72.5	46.5	48	32	43	42	32.5	30.5	26.5	39.5
Chloramphenicol	15	3	49.5	45.5	70	75	58	57	52.5	59	57.5	49.0
Kanamycin	42.5	17	66.5	88	100	98	99	96	100	96.5	97.5	81.5
Streptomycin	60	48	90.5	100	100	96	100	95	99	99	100	89.0
Sulphonamide	78	72	92.5	100	100	100	100	100	99	100	100	94.0
Tetracycline	45.5	18	96	96.5	100	100	100	98	97	93	100	82.5
No. of strains of <i>E. coli</i> examined	99	100	95	90	100	100	100	100	99	85	87	1055

Source: from Linton, Timoney and Hinton (1981).

^a Numbers to nearest 0.5%.

calves. This percentage was not dissimilar to published data (Howe and Linton, 1976; Jackson, 1981). During the course of their stay on the farm, the proportion of the calf isolates resistant to chloramphenicol rose to 75% on day 15 and later levelled out to 57.5% on day 34 (*Table 45.2*). The R-determinants Cm, Sm, Su, Tc were present in 461 (89%) of all chloramphenicol-resistant *E. coli* isolated. This combination of R-determinants is similar to those carried on the H2 incompatibility R-plasmid of the *S. typhimurium* phage type DT 193 causing infection in the same calves, suggesting possible exchange of R-plasmids. The fact that this combination of R-determinants was found in 37 O-serogroups of *E. coli* suggested that multiple transfers between *E. coli* had occurred.

Evidence that transfer between the *Salmonella typhimurium* and the *E. coli* had occurred at some time or other was sought by looking at the properties

of the plasmids carrying chloramphenicol resistance. The R-determinant for chloramphenicol resistance in the *S. typhimurium* phage DT 193 was carried, along with those for Sm, Su and Tc, on an H2 incompatibility plasmid (Threlfall, Ward and Rowe, 1978a; 1978b). Transfer of H2 incompatibility plasmids is thermosensitive; it occurs at 28°C, but not at 35°C (Smith, 1974; Anderson, 1975; Terakado and Sato, 1978). The proportion of chloramphenicol-resistant *E. coli* carrying an H2 incompatibility plasmid was determined in 146 representative strains containing R-determinants for Cm, Sm, Su, Tc. These were selected at random from strains isolated on non-selective media. Forty-eight per cent exhibited transfer of all or part of their complement of R-determinants at both 35°C and 28°C; this indicated the presence of many plasmids whose transfer was not thermosensitive. However, in 11% of isolates transfer occurred only at 28°C and included the four R-determinants characteristic of the H2-plasmid in *S. typhimurium*. These were confirmed as H2-plasmids by their incompatibility with the standard H2-plasmid (TP116) and by their resistance to tellurite and mercury. Strains carrying H2-plasmids represented 5 O-serogroups and non-typable strains. Although H2-plasmids have been found in *Citrobacter*, *Serratia*, *Klebsiella*, *Shigella* and *Escherichia* (Taylor and Grant, 1977a; 1977b; Smith, Parsell and Green, 1978), they are relatively uncommon in these genera. The occurrence of H2-plasmids in 11% of the representative *E. coli* strains in the study described above is, therefore, unusually high and suggests that special circumstances were involved in this situation. The epidemic of chloramphenicol-resistant *S. typhimurium* in the calf industry, together with the selective pressures resulting from the wide use of many antibiotics on the rearing farm, was probably responsible for this occurrence.

As transfer of H2-plasmids is thermosensitive, it is unlikely to occur *in vivo*. Studies were therefore undertaken to elucidate the ecology of H2-plasmids and to determine how the calf intestine became colonized by strains carrying these plasmids.

To test the hypothesis that the temperature of the calf body would preclude transfer occurring, calves were given both donor *E. coli* (carrying the H2-plasmid) and a marked recipient strain, i.e. nalidixic acid-resistant (Timoney and Linton, 1982). *In vivo* transfer of the H2-plasmid was not detected in groups of calves which had been muzzled and so denied oral contact with their surroundings. Transfer to the marked recipient, and to a number of other calf *E. coli* strains, was observed in calves not muzzled or from which muzzles were removed during the experiment. Also transfer of the H2-plasmid was demonstrated in voided faeces held at 28°C, but not at 37°C. These results indicated that H2-plasmids did not transfer in the gut, but were most probably acquired by the calf from its environment where conjugation had taken place.

Further to these observations, it was noted that studies on calves fed isogenic strains of *E. coli*, with and without the H2-plasmid, demonstrated that the strain carrying the plasmid persisted for a longer time in the intestine than the one not carrying the plasmid (Figure 45.2). This occurred in the absence of an antibiotic selective pressure and suggested that the H2-plasmid carried genes which enhanced the ability of strains carrying the plasmid to maintain themselves in the gut. The presence of this plasmid in the strains of multiply-resistant strains of *S. typhimurium* could therefore play a role in

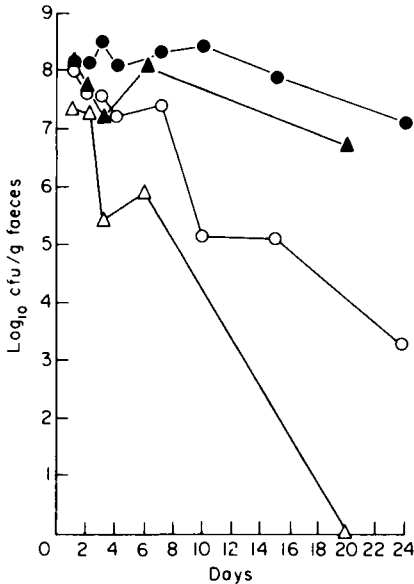


Figure 45.2 Effect of the presence of an H2-plasmid on the persistence of two strains (021 and 045) of *Escherichia coli* in the intestines of calves. Each point for the 021 and 045 isogenic strains represents the mean counts from 8 and 4 calves, respectively. On day 0, each calf was given equal numbers of the strain with and without the H2 plasmid (●, 045 with plasmid; ○, 045 without plasmid; ▲, 021 with plasmid; △, 021 without plasmid) (from Timoney and Linton, 1982)

their greater virulence and epidemicity. Other workers have reached the same conclusion (van Leeuwen, Voogd and Guinée, 1981).

In these studies, continual fluctuations in the range of *E. coli* O-serogroups in the dominant gut flora was demonstrated. This variation occurred both qualitatively and quantitatively, and resembled similar studies in pigs (Linton, Handley and Osborne, 1978). The selection pressures of the variety of antibiotics used on the farm must have had some effect in deciding the dominant O-serogroups. As most isolates carried a wide range of R-determinants, any of the drugs used on the farm could have selected for the multiply-resistant *E. coli*. On the other hand, recent ecological studies on *E. coli* in calves kept under conditions of dairy replacement, as well as intensive systems, have shown as rapid a turnover of O-serogroups in the absence of any antibiotic selection pressure (unpublished data).

Antibiotic-resistant bacteria in calf slurry and the calf's environment

In the past decade there has been considerable interest in the survival of pathogenic microorganisms in slurry (a mixture of faeces, urine and water from intensive systems of husbandry where bedding is not used), and their potential danger as a source of infection for farm stock and, indirectly in food and water, for man (Jones, 1980). On the other hand, there is little published information on the survival of microorganisms containing R-plasmids in slurry and in the environment, following the spreading of slurry on farm land

(Kelly and Collins, 1978). Accordingly, studies were undertaken on the survival of naturally occurring *S. typhimurium* and multiple-antibiotic resistant *E. coli* in slurry (Hinton and Linton, 1982), on pasture treated with slurry and in the animals' environment.

SURVIVAL OF *Salmonella typhimurium* AND ANTIBIOTIC-RESISTANT *Escherichia coli* IN STATIC SLURRY

Slurry was obtained from the veal calf unit described above. A bulk sample was stored in a polyethylene-lined galvanized dustbin and sampled, after thorough mixing, on 8 occasions over a period of 7 weeks (Hinton and Linton, 1982).

Viable *S. typhimurium* persisted in small numbers (10–100/ml) in stored static slurry throughout the 7-week period of observation. This was not unexpected, since salmonellae have been shown to persist for nearly 300 days in slurries of farm animal origin (Jones, 1980). The small numbers of *S. typhimurium* recorded were unlikely to pose a significant threat to farm stock grazing fields dressed with this slurry, since it has been shown that calves become infected with *S. dublin* only following the grazing of pasture 18 h after the application of a slurry containing 10^6 *S. dublin*/ml, but not one containing only 10^3 /ml (Taylor and Burrows, 1971).

The number of viable coliforms in the slurry fell in the early period of storage, although the population remained reasonably stable thereafter. This suggested that the majority of *E. coli* were either preserved by the cold (the study was carried out in the winter) or the population was maintained by the *E. coli* multiplying at a rate sufficient to replace those that were dying.

Analysis of the structure of the dominant *E. coli* flora, using O-serotyping and biotyping, indicated that certain components of the population remained relatively stable during the 7-week period, since the most common O-sero-group/biotype combinations were isolated from more than half of the samples examined. Nevertheless, the composition of the total *E. coli* population was extremely complex, since not only were a relatively large number of O-serogroups identified (48), but the distribution of R-determinants gave a wide range of different antibiotic-resistance patterns among many of these O-serogroups.

A disturbing feature arising from this investigation was the high level of chloramphenicol resistance among the *E. coli*, although the incidence was far from uniform among the dominant *E. coli* O-serogroups and was not detected in several of them. Interestingly, the proportion of the *E. coli* exhibiting chloramphenicol resistance (55%) was very similar to that observed in *E. coli* faeces isolated from the faeces of calves reared on the farm.

The intensive nature of veal production, coupled with the use of antibacterial drugs for therapeutic purposes, favoured the maintenance of a high level of chloramphenicol resistance among the *E. coli* flora of the calves on this farm, since chloramphenicol is always associated with multiple drug resistance. This fact, coupled with protracted survival of the *E. coli* in the slurry, suggests that farms of this type present a potential reservoir of plasmids coding for chloramphenicol resistance in the environment, even when this drug is never prescribed either for prophylaxis or the treatment of diseased calves.

SURVIVAL OF *Escherichia coli* ON PASTURE TREATED WITH SLURRY

Preliminary studies were carried out using slurry from cattle and pig houses spread separately on ungrazed pasture at a loading similar to normal spreading procedures (1 l of slurry was spread evenly over 1 m² of pasture). Representative samples of upper grass (15–20 cm) above soil and lower grass (including roots and some topsoil) were collected using sterile scissors and gloved hands. Samples of 20 g were washed with 100 ml nutrient broth and viable coliforms counts determined, the counts being expressed as numbers of coliforms/g of sample. Typical results are shown in *Figures 45.3(a)* and *45.3(b)*.

The coliform counts on upper grass treated with the cattle slurry (*Figure 45.3a*) showed a rapid fall over the first week (from 10⁶/g on day 1 to 10⁴/g on day 7). No change was noted over the next week, but counts then fell rapidly to 10²/g between days 14 and 20. After 6 weeks, no coliforms could be detected.

The lower grass showed a slower decrease, falling by 1 log. over the first 14

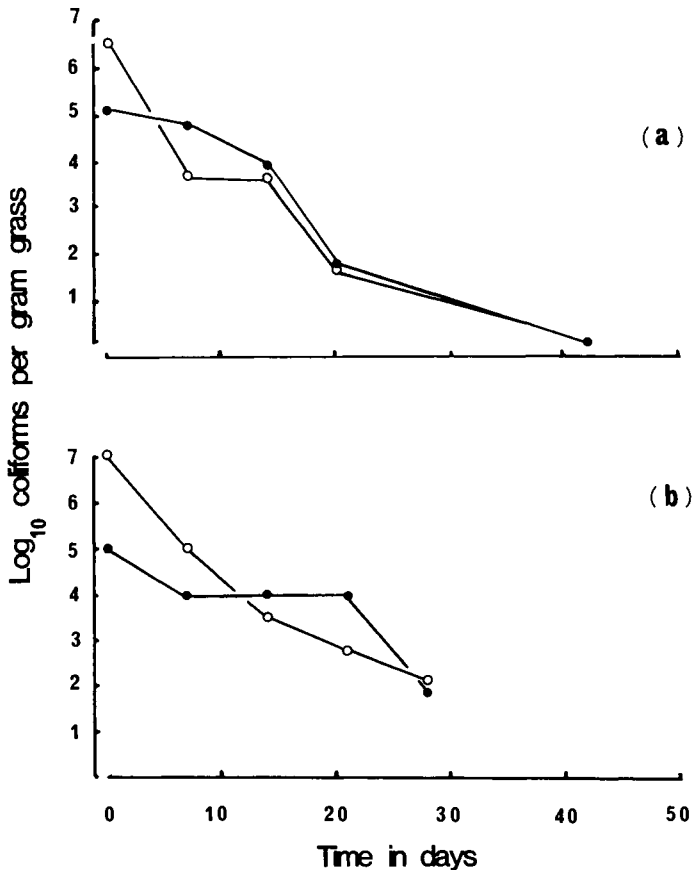


Figure 45.3 Numbers of viable coliforms persisting with time on pasture spread with slurry (a) from calves and (b) from pigs (○, coliform counts on upper grass, 15 cm or higher; ●, coliform counts on lower grass, including roots)

days. This was followed by a more rapid death rate over the next 7 days from 10^4 /g to 10^2 /g. Coliforms could not be detected on day 42.

Counts on upper grass samples taken from pasture treated with pig slurry demonstrated almost a linear fall in numbers over 30 days, falling from 10^7 /g to 10^2 /g (Figure 45.3b). Counts on the lower grass showed little decline over the first 21 days (remaining at about 10^4 /g), then a more rapid decline over the next 7 days falling to 10^2 /g.

These findings are in agreement with those of Taylor and Burrows (1971) in that *E. coli* from cattle slurry survived longer in lower grass. There is little difference between counts on upper and lower grass treated with pig slurry. It must be stressed that these experiments were carried out in mid-winter, at around 0°C throughout, with only intermittent sunshine. No doubt more dramatic rates of decline would have been recorded in summer. Nevertheless, duration of antibiotic-resistant coliform was limited in numbers and time, and treated pasture left ungrazed for 6 weeks is not likely to constitute an important source of antibiotic-resistant coliforms for animals.

Antibiotic-resistant bacteria (other than *Escherichia coli*) isolated from slurry and the animals' environment

As stated earlier, the majority of studies on antibiotic-resistant bacteria in calves has concentrated on *Salmonella* spp. and *E. coli*. No doubt this was due, at least in part, to the relative ease of characterizing these genera and biotyping strains. However, a wider appraisal of the antibiotic resistance status of bacteria associated with the calf and its environment is needed. Preliminary studies on cow slurry and the environment of the calf, on a conventional dairy farm, have been carried out.

Table 45.3 ISOLATES FROM COW SLURRY OF ANTIBIOTIC-RESISTANT BACTERIA (OTHER THAN *Escherichia coli*)

Organism	Resistance determinants ^a
<i>Pseudomonas aeruginosa</i>	Cm Sm Su Tc Ap Km (1) ^b
<i>Pseudomonas fluorescens</i>	Cm Sm Su Tc Ap Tp (5)
<i>Alkaligenes</i> spp.	Cm Sm Su Tc Ap Km Tp (1)
	Sm Su Tc Km Tp (3)
<i>Proteus vulgaris</i>	Sm Su Tc Km Tp (5)
	Cm Sm Su Tc Km Tp (1)
<i>Proteus morgani</i>	Cm Sm Su Tc Ap Km Tp (1)
	Sm Su Tc Km Tp (3)
<i>Providencia alcalifaciens</i>	Cm Sm Su Tc Km (2)
	Sm Su Tc Km Tp (2)
<i>Aeromonas hydrophila</i>	Cm Sm Su Tc Km Tp (1)
	Sm Su Tc Ap Km Tp (4)
<i>Acinetobacter calcoaceticus</i>	Su Tc Km Tp (1)
	Sm Su Tc Tp (4)
<i>Hafnia</i> sp.	Sm Su Tc Tp (1)

^a Cm, chloramphenicol; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; Tp, trimethoprim.

^b Numbers of isolates in parentheses.

COW SLURRY

Isolates of non-lactose-fermenting bacteria from cow slurry were identified and their antibiotic resistance patterns determined (*Table 45.3*). Virtually all isolates of a wide range of genera and species were multi-resistant and preliminary mating experiments with standard *E. coli* K12 recipients have shown that at least some of the R-determinants could be transferred at one or more temperatures (22°C, 28°C or 37°C) from the majority of isolates. The plasmid profiles of these isolates has yet to be determined.

CALF ENVIRONMENT

Relatively few non-lactose-fermenting bacteria are isolated regularly on bile lactose agar from calf faeces and since many had been demonstrated in slurry it was assumed that they were derived from the environment; the flora of the calf's environment therefore was studied. Preliminary studies only have been carried out on bedding samples from calf pens. Samples were collected weekly over a 6-week period. The bedding (visually free of solid faeces) was shaken in broth and the resultant suspension plated onto bile lactose agar both unsupplemented and supplemented with one of several antibiotics. The results are shown in *Table 45.4*.

A range of non-lactose-fermenting bacteria were isolated including *Aerococcus* sp., *Acinetobacter* spp. and *Proteae*. *Table 45.4* presents the antibiotic resistance pattern of 45 isolates of *Acinetobacter* spp. isolated on unsupplemented bile lactose agar. All isolates were resistant to certain antibiotics and

Table 45.4 ANTIBIOTIC RESISTANCE DEMONSTRATED IN *Acinetobacter* spp. AND *PROTEAE* ISOLATED FROM BEDDING IN CALF PENS

Organism	Resistance determinants ^a
<i>Acinetobacter</i> spp.	Su Tc (4) ^b Su Tp (3) Su Tc Sp (4) Su Tp Sm (9) Su, Tc Km (2) Su Tc Tp Sm (2) Su Tp Sp Sm (8) Su Tp Cm Sm (1) Su Tc Tp Km (1) Su Tc Tp Sp (2) Su Tc Tp Km Sm (7) Su Tc Tp Sp Sm (1) Su Tc Tp Sp Km Cm Sm (1) <i>Proteus mirabilis</i> Su Tc Tp Ct Fz (3) Su Tc Tp Ct Fz Ap Cm Sp Sm (1) <i>Providencia stuartii</i> Tc Ct Fz Sp (1) <i>Proteus vulgaris</i> Su Tc Tp Ct Cm Sm Sp (2) Su Tp Ct Sm Sp (1) Su Tp Ct Cm Sm Sp (1) Su Tp Ap Cm (1)

^a Symbols as in first footnote to *Table 45.3*. Additional symbols: Ct, colistin sulphate; Sp, spectinomycin; Fz, furazolidone.

^b Numbers of isolates in parentheses.

different strains exhibited up to 7 R-determinants. Table 45.4 also includes data on members of the Proteae. These were isolated on supplemented and unsupplemented bile lactose agar and demonstrated a similar, wide range of resistance patterns. Transfer studies and R-plasmid profiles on these isolates have yet to be investigated.

It is obvious that a wide range of R-determinants are carried by many non-lactose-fermenting members of the flora, as well as by *E. coli*, in the calf and its environment.

Plasmid profiles of *Escherichia coli* and chloramphenicol-resistant *Salmonella typhimurium*

The ecological studies described so far have been based on O-serogrouping, biotyping and resistogram typing of bacterial isolates. This has yielded much useful information, but strains with similar characters, even where demonstrating the same antibiotic-resistance patterns, may in fact be carrying different R-plasmids. Where R-plasmids have been characterized, as in *Salmonella* (Threlfall, 1981; O'Brien *et al.*, 1982); *E. coli* (Levy, FitzGerald and Maccone, 1976; Linton *et al.*, 1977; Casewell, 1982), valuable epidemiological data on the ecology of plasmids and their host bacteria has emerged.

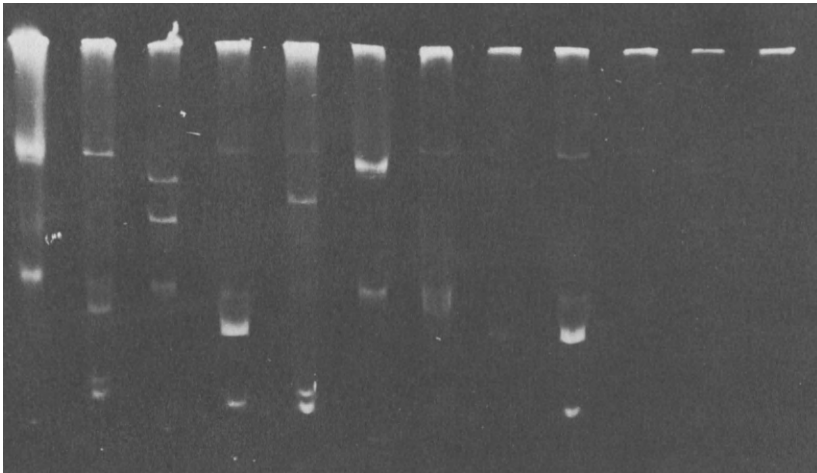
To extend the usefulness of our own data, it was decided to study the plasmid profiles of selected strains. Our preliminary observations are reported below.

Two batches, each of 70 strains of *E. coli*, were isolated in May 1981 (Nos 1–70) and September 1982 (Nos 71–140), respectively, from slurry obtained from the intensive veal calf unit, previously described, where endemic chloramphenicol-resistant *S. typhimurium* had been regularly experienced (Linton, Timoney and Hinton, 1981; Hinton *et al.*, 1983). All isolates of *E. coli* selected were chloramphenicol resistant and multiple resistant. All strains were O-serogrouped, biotyped and resistogram typed. In addition, the plasmid profile of each was determined by a modified technique of Birnboim and Doly (1979). This agarose gel electrophoresis technique was developed initially to study the plasmid profiles of *E. coli* K12 transconjugants. The technique was modified for use with wild strains of *E. coli* (P. Bennett, unpublished) and can be used for the rapid study of large numbers of isolates of different genera and species.

Using the plasmid profiles obtained on each of the 140 *E. coli* isolates, it was possible to draw a number of important conclusions on the relationship of these strains.

Figures 45.4 and 45.5 present plasmid profiles of a selection of isolates, representing a number of O-serogroups and non-typable strains, isolated in May 1981. Distinct profiles were found in different O-serogroups and between non-typable strains. The fact that many of the non-typable strains were of different biotypes indicated that they were not closely related. These findings suggest that strains of different serogroup or biotype are likely to carry different plasmids.

On the other hand, within the same O-serogroup—Figure 45.4, strains 20 and 51 (0159); Figure 45.5, strains 60 and 62 (087); 21, 23 and 65 (0113)—



Strain No. ^a	13	50	25	45	11	34	20	28	42	51	32	37
O-sero group	NT	NT	NT	NT	NT	NT	159	NT	NT	159	8	162
Biotype	22	30	19	23	21	22	19	23	23	19	6	19
Sm ^b	R ^c	R	R	R	R	R	R	R	R	R	R	R
Tc	R	R	R	R	R	R	R	R	R	R	R	R
Cm	R	R	R	R	R	R	R	R	R	R	R	R
Km	R	R	R	R	R	R	R	R	R	R	R	R
Ap	R	S	R	S	S	R	S	S	S	S	S	R
Su	R	R	R	R	R	R	R	R	R	R	R	R

^a A number of these strains carried additional small plasmids which were not clearly resolved on the lower part of the gel and, for this reason, that part of the gel has not been included.

^b See first footnote to Table 45.3 for abbreviations.

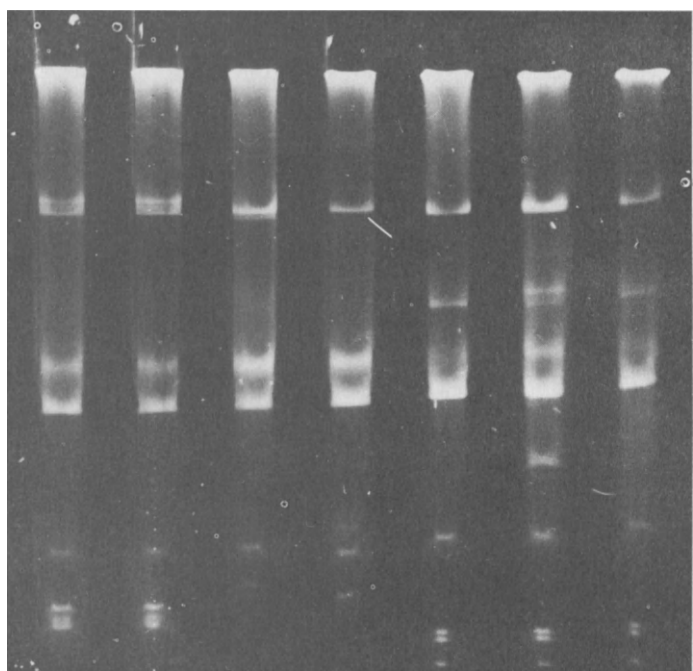
^c R, resistant; S, sensitive.

Figure 45.4 Plasmid profiles of 12 strains of different O-serogroups and non-typable isolates of *Escherichia coli* from veal calf slurry in May 1981

similar plasmid profiles were found. This was confirmed by running 2 groups of O-serogroups (020 and 08) on the same gel (Figure 45.6).

By comparing the plasmid profiles of strains of the same O-serogroup isolated from both batches of slurry, it was possible to determine whether or not their plasmid carriage remained unchanged over a 15-month period. Of all the O-serogroups isolated from both batches of slurry, 020 was most common. Strain 53 (020) from the first batch was run in parallel with isolates from the second (Figures 45.7 and 45.8). In Figure 45.7, strain 104 (020) demonstrated some differences compared with strain 53 (020), whereas strain 107 (020) was very similar. In Figure 45.8, strains 114, 118 and 119 (020) showed similar profiles to strain 53. It is concluded that in some strains of the same O-serogroup isolated from a common ecological niche, the plasmid profile remained substantially the same over a 15-month period; in a few strains differences, in loss or gain of a plasmid, were noted.

One particular question required an answer: how similar are the plasmid profiles in *E. coli* to those in the chloramphenicol-resistant *S. typhimurium*? So far, wild strains of *S. typhimurium* have not been examined, but transcon-

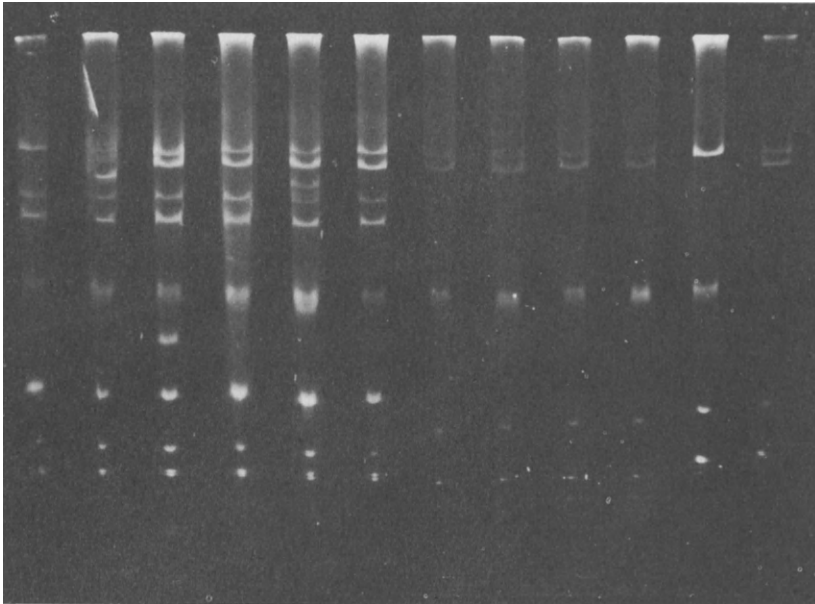


Strain No.	60	62	3	26	21	23	65
O-serogroup	87	87	101	111	113	113	113
Biotype	22	22	30	22	24	10	24
Sm ^a	R ^b	R	R	R	R	R	R
Tc	R	R	R	R	R	R	R
Cm	R	R	R	R	R	R	R
Km	R	R	R	R	R	R	R
Ap	R	R	R	S	R	R	R
Su	R	R	R	R	R	R	R

^a See first footnote to *Table 45.3* for abbreviations.

^b R, resistant; S, sensitive.

Figure 45.5 Plasmid profiles of 7 strains of *Escherichia coli*, isolated from veal calf slurry in May 1981, showing similarities within O-serogroups



Strain No.	10	12	41	53	31	66	1	2	4	15	18	19
O-sero group	20	20	20	20	20	20	8	8	8	8	8	8
Biotype	22	22	22	22	22	22	6	6	6	6	6	6
Sm ^a	R ^b	R	R	R	R	R	R	R	R	R	R	R
Tc	R	R	R	R	R	R	R	R	R	R	R	R
Cm	R	R	R	R	R	R	R	R	R	R	R	R
Km	R	R	R	R	R	R	R	R	R	R	R	R
Ap	R	R	R	R	R	R	S	S	S	S	R	R
Su	R	R	R	R	R	R	R	R	R	R	R	R

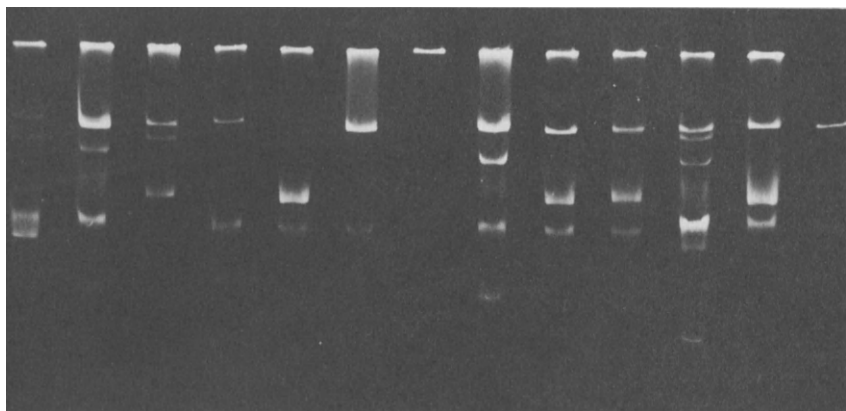
^a See first footnote to Table 45.3 for abbreviations.

^b R, resistant; S, sensitive.

Figure 45.6 Profiles of 12 strains of 2 O-serogroups of *Escherichia coli* isolated from veal calf slurry in May 1981

jugants in *E. coli* K12 were run on the far right of some of the gels (Figures 45.7 and 45.8). The plasmid profile of *S. typhimurium* appears to be much simpler than in certain strains of *E. coli*, but a single band, above that of chromosomal DNA, corresponds with one of the two bands in the same region of strains of *E. coli* 020 (Figure 45.6, strains 53, 104 and 107; Figure 45.8, strains 114, 118 and 119).

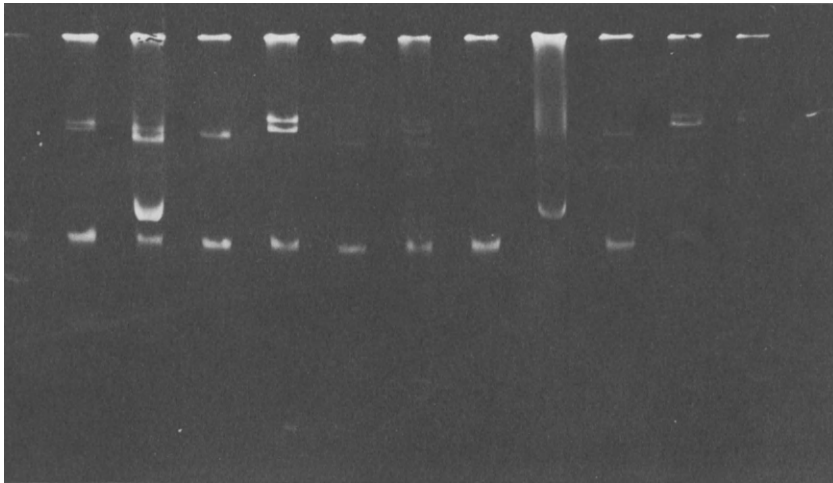
These preliminary observations indicate the value of plasmid profiles in epidemiological studies. With this technique now available it will be possible to study the profiles of large numbers of isolates, including antibiotic-resistant species of other genera associated with the animal and its environment.



Strain No.	53	95	96	97	98	100	102	103	104	106	107	108	SS2 ^a
O-serogroup	20	13	NT	130	NT	101	40	NT	20	NT	20	153	K12
Biotype	22	19	19	15	23	19	31	30	30	30	22	30	
Sm ^b	R ^c	R	R	R	R	R	R	R	R	R	R	R	R
Tc	R	R	R	R	R	R	R	R	R	R	R	R	R
Cm	R	R	R	R	R	R	R	R	R	R	R	R	R
Km	R	R	R	R	R	R	R	R	R	R	R	R	S
Ap	R	S	S	S	S	R	R	R	R	S	S	S	S
Su	R	R	R	R	R	R	R	R	R	R	R	R	R

^a Transconjugant of *S. typhimurium* phage type DT204c in *E. coli* K12 selected on bile lactose agar supplemented with chloramphenicol.
^b See first footnote to *Table 45.3* for abbreviations.
^c R, resistant; S, sensitive.

Figure 45.7 Profiles of strains of *Escherichia coli* isolated from the veal calf unit in May 1981 and September 1982, and a transconjugant of *Salmonella typhimurium*



Strain No. ^a	53	109	110	111	112	113	114	115	116	117	118	119	SS2 ^b
O-serogroup	20	NT	9	1	NT	NT	20	NT	9	15	20	20	K12
Biotype	22	28	19	26	22	28	22	30	19	22	30	22	
Sm ^c	R ^d	R	R	R	R	R	R	R	R	R	R	R	R
Tc	R	R	R	R	R	R	R	R	R	S	R	R	R
Cm	R	R	R	R	R	R	R	R	R	R	R	R	R
Km	R	R	R	R	R	R	R	R	R	R	R	R	S
Ap	R	S	R	R	R	S	S	S	R	S	S	S	S
Su	R	R	R	R	R	R	R	R	R	R	R	R	R

^a A number of these strains carried additional small plasmids which were not clearly resolved on the lower part of the gel and, for this reason, that part of the gel has not been included.

^b See first footnote to Figure 45.7.

^c See first footnote to Table 45.3 for abbreviations.

^d R, resistant; S, sensitive.

Figure 45.8 Profiles of strains of *Escherichia coli* isolated from veal calf slurry in May 1981 and September 1982, and a transconjugant of *Salmonella typhimurium*

Acknowledgements

We are deeply indebted to Dr P. Bennett (Department of Microbiology, University of Bristol) for permission to quote unpublished work on the plasmid profiles of *Escherichia coli*. Also, we wish to express our thanks to our technical staff without whose dedication these extensive studies would not have been possible.

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